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XXXIV. ON THE CHANGE OF THE OSMOTIC PRESSURE OF SOLUTIONS OF CERTAIN COLLOIDS UNDER THE INFLUENCE OF SALT SOLUTIONS.

By DAIZO OGATA (*Fukuoka*).

From the Department of Biochemistry, Oxford University.

(*Received March 17th, 1922.*)

In spite of certain controversies among investigators on the osmotic pressure exerted by colloidal solutions the positive proof that a definite, though small, osmotic pressure is set up was given by Starling [1896], Moore and Parker [1902], Lillie [1907], Moore and Roaf [1907].and others.

This osmotic pressure is affected by several factors, such as the mode of the formation of the colloidal solution [Lillie, 1907], the change of temperature [Moore and Roaf, 1907], the acidity or alkalinity of solution [Adamson and Roaf, 1908]. Further that the added salts also increase or diminish the size of the colloidal particles before any visible precipitation takes place, *i.e.* lower or raise the osmotic pressure, has been proved by Biltz and Vegesack [1909], Lillie [1907], Moore and Roaf [1907] and Loeb [1918].

The present research was started, by suggestion of Prof. Moore and under his direction, to prove by means of osmotic pressure measurements whether, first, salt solutions affect the "solution aggregate" of different colloidal solutions always in the same way, and, secondly, what is the influence, if any, of certain anaesthetics upon colloidal solution. While definite and regular results were obtained in the former experiments, those in the latter were not at all decisive.

Methods.

As colloidal solutions gum arabic (1 %), egg albumin (5 %) and sheep serum (half diluted) were used. The membrane (dialyser) was of collodion and made on the outside of a large test tube. This was fixed to a well-fitting rubber cork with a strong string. The cork had two holes, one for a glass manometer tube and the other for an inlet and outlet tube. The length of the membrane was so adjusted as to limit its content to approximately 15 cc.

In the earlier part of the experiments the colloidal solution only was introduced into the dialyser, *i.e.* without mixing previously with any salt solution, thus letting the latter penetrate gradually from the outside. But

the procedure became more elaborate with the progress of the experiments, and the final method consisted of mixing the colloidal solution with the salt solution just before setting up the apparatus and then introducing into the membrane. The membrane was immersed up to the upper surface of the cork in a definite volume (120 cc. as a rule) of salt solution contained in a beaker. The manometer tube, together with a millimetre scale, was clamped in a vertical position during the experiments. The salts employed were copper sulphate, calcium chloride and alkaline sodium phosphate respectively.

The experiments were performed in series, *e.g.*, 1/10 *N*, 1/100 *N*, 1/1000 *N* solution and solvent (distilled water) only, the last as a control. The same salt solution of the same concentration was always placed inside and outside the membrane.

Usually the osmotic pressure was not very high, therefore the consideration of the distension of the membrane was of little importance.

As the salt solution could easily pass through the membrane the column of solution in the manometer increased in length, at first, very rapidly. After a certain time it began to fall, hardly staying at the highest point any measurable length of time. Then the column fell very slowly to a certain level at which it remained for a considerable time. When almost the same level was kept for at least two days, it was understood, in my experiments, that the expected equilibrium of osmotic pressure was attained. In most cases when this equilibrium was reached the outside liquids were replaced with distilled water, four or five times in succession with an interval of one day or more. The reversibility of pressure was fairly satisfactory.

Results of Experiments.

The following are the examples of the abridged records of experiments carried out during five months. Similar results were always obtained when due precautions were taken.

Experiments with gum arabic solution. (See Fig. 1.)

Inside the membrane: 1 % gum arabic solution, 15 cc.
Outside the membrane: salt solutions of different concentrations, 120 cc.

Table I. SO_4Cu.

Day	Hour	Temp.	Osmotic pressure			
			1/10 *N* SO_4Cu	1/100 *N* SO_4Cu	1/1000 *N* SO_4Cu	distilled water
Sept. 1	afternoon (set up)					
„ 2	4.00 p.m.	18·9° C.	2·5 mm.	13·2 mm.	48·0 mm.	163·3 mm.
„ 3	2.30	16·9	7·5	14·0	45·1	150·5
„ 5	4.00	18·9	8·5	12·8	42·0	135·5
„ 6	3.20	18·3	9·0	13·0	42·2	130·0
p_H of the outside solution, about			9·0	7·2	6·5	6·5
The reversibility was tested:						
Sept. 13	10.00 a.m.	17·3	92·0	100·0	106·0	120·0

The curves in Fig. 1 indicate the rate at which equilibrium was reached both in direct and "reversed" experiments.

Table II. Cl_2Ca.

Day	Hour	Temp.	Osmotic pressure: 1/10 *N* Cl_2Ca	1/100 *N* Cl_2Ca	1/1000 *N* Cl_2Ca	distilled water
Sept. 2	about noon (set up)					
„ 6	3.30 p.m.	17·5° C.	12·3 mm.	17·0 mm.	50·0 mm.	108·5 mm.
p_H of the outside solution, about			6·0	6·5	6·0	6·5
The reversibility was tested:						
Sept. 13	10.00 a.m.	16·9	93·0	94·0	113·0	117·5

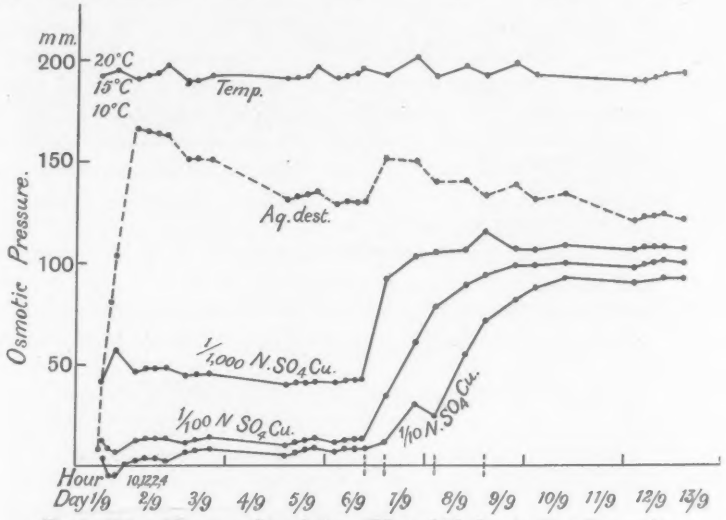

Fig. 1. Exp. with gum arabic solution. Effect of SO_4Cu. At the ⋮ line on the abscissa, the outside liquid was replaced by dist. water in succession.

Table III. PO_4HNa_2.

Day	Hour	Temp.	Osmotic pressure: 1/10 *N* PO_4HNa_2	1/100 *N* PO_4HNa_2	1/1000 *N* PO_4HNa_2	distilled water
Sept. 3	morning (set up)					
„ 7	12.00	17·2° C.	15·2 mm.	27·0 mm.	110·5 mm.	149·0 mm.
p_H of the outside solution, about			9·0	7·2	6·5	6·5
The reversibility was tested:						
Sept. 13	10.00 a.m.	17·2	251·0	220·0	129·0	130·0

Experiments with egg albumin.

Inside the membrane: 5 % egg albumin solution, 15 cc.
Outside the membrane: salt solutions of different concentrations, 120 cc.

Table IV. SO_4Cu.

Day	Hour	Temp.	Osmotic pressure: 1/2000 *N* SO_4Cu	1/20,000 *N* SO_4Cu	1/200,000 *N* SO_4Cu	distilled water
Sept. 16	morning (set up)					
„ 20	12.00	16·1° C.	12·0 mm.	53·9 mm.	55·1 mm.	52·2 mm.
p_H of the outside solution, about			6·0	6·0	6·0	6·0
The reversibility was tested:						
Sept. 27	4.00 p.m.	16·0	36·0	64·0	62·0	59·0

Table V. Cl_2Ca.

Day	Hour	Temp.	Osmotic pressure			
			1/20 *N* Cl_2Ca	1/200 *N* Cl_2Ca	1/2000 *N* Cl_2Ca	distilled water
Sept. 16	afternoon (set up)					
,, 20	12.00	15·7° C.	14·0 mm.	17·0 mm.	36·0 mm.	50·0 mm.
p_H of the outside solution, about			6·75	6·0	6·0	6·0
The reversibility was tested:						
Sept. 27	4.00 p.m.	15·5	40·0	41·0	49·0	58·0

Table VI. PO_4HNa_2.

Day	Hour	Temp.	Osmotic pressure			
			1/20 *N* PO_4HNa_2	1/200 *N* PO_4HNa_2	1/2000 *N* PO_4HNa_2	distilled water
Sept. 16	afternoon (set up)					
,, 21	2.00 p.m.	16·5° C.	22·6 mm.	30·0 mm.	44·0 mm.	47·0 mm.
p_H of the outside solution, about			9·0	7·2	6·75	6·5
The reversibility was tested:						
Sept. 27	4.00 p.m.	15·9	40·0	48·0	51·0	59·0

Experiments with blood serum.

Inside the membrane: sheep serum half diluted, 15 cc.
Outside the membrane: salt solutions of different concentrations, 120 cc.

Table VII. SO_4Cu.

Day	Hour	Temp.	Osmotic pressure			
			1/2000 *N* SO_4Cu	1/20,000 *N* SO_4Cu	1/200,000 *N* SO_4Cu	distilled water
Sept. 30	afternoon (set up)					
Oct. 5	12·00	16·6° C.	161·0 mm.	189·0 mm.	192·0 mm.	184·0 mm.
p_H of the outside solution, about			6·75	7·2	7·2	7·2
Sulphate in 100 cc. of the outside solution as SO_3			5·36 mg.	2·86 mg.	0·06 mg.	0·00 mg.
The reversibility was tested:						
Oct. 17	10.00 a.m.	13·4	115·0	168·0	163·0	170·0

Table VIII. Cl_2Ca.

Day	Hour	Temp.	Osmotic pressure			
			1/20 *N* Cl_2Ca	1/200 *N* Cl_2Ca	1/2000 *N* Cl_2Ca	distilled water
Sept. 30	afternoon (set up)					
Oct. 5	12.00	16·0° C.	105·0 mm.	120·0 mm.	169·0 mm.	176·0 mm.
p_H of the outside solution, about			7·2	7·2	7·2	7·2
Chloride in 100 cc. of the outside solution as ClNa			37·7 mg.	11·5 mg.	9·25 mg.	8·0 mg.
The reversibility was tested:						
Oct. 17	10.00 a.m.	13·1° C.	178·0 mm.	168·5 mm.	155·0 mm.	163·0 mm.

Table IX. PO_4HNa_2.

Day	Hour	Temp.	Osmotic pressure			
			1/20 *N* PO_4HNa_2	1/200 *N* PO_4HNa_2	1/2000 *N* PO_4HNa_2	distilled water
Oct. 1	morning (set up)					
,, 5	12.00	16·5° C.	133·5 mm.	163·0 mm.	166·0 mm.	176·0 mm.
p_H of the outside solution, about			9·0	7·2	6·75	6·75
Phosphate in 100 cc. of the outside solution as P_2O_5			204·0 mg.	27·2 mg.	8·0 mg.	3·0 mg.
The reversibility was tested:						
Oct. 17	10.00 a.m.	13·2° C.	69·0 mm.	101·0 mm.	137·0 mm.	158·0 mm.

Unless specially mentioned the protein did not appear in the outside solution through the membrane (acetic acid and potassium ferrocyanide test).

As clearly seen in the above tables the salts used have all a more or less prominent influence upon the osmotic pressure exerted by the colloidal solutions such as gum arabic, egg albumin or blood serum. The salt solutions always reduced the osmotic pressure of the colloidal solution, and the more dilute the salt solution, the less decrease was brought about, thus gradually approaching the control with distilled water only. This fact suggests, at once, the important influence of electrolytes, because within the limits of concentration of the salt solutions employed here it may safely be concluded that the more concentrated the solution, the greater will be the number of electrically dissociated molecules. The trouble is the appearance of precipitation in the long run of experiments of this kind, and, therefore, all the statements here are based necessarily on the comparison with the control otherwise under the same conditions. Of copper sulphate an extremely dilute solution was made use of simply to avoid the unnecessary precipitation as far as possible.

The temperature, no doubt, affects the height of the osmotic pressure, but this effect is rather slight compared with that due to the electrolytes themselves. Notwithstanding the fluctuation of osmotic pressure parallel to that of temperature, the main course of change of osmotic pressure cannot be ignored.

The hydrogen-ion is neither the sole nor the principal cause in these cases, since the osmotic pressure does not always keep pace with its concentration. To quote a few examples, the hydrogen-ion concentration was about the same through the series in Tables II, IV, VII and VIII respectively. Nevertheless the pressure difference is very notable.

The more or less prominent reversibility of the pressure leads us to seek the probable cause in the physical reaction rather than the chemical change, as was propounded by several previous investigators. Mayer, Schaeffer and Terroine [1907] observed under the microscope that the submicroscopical granules of colloidal solutions (metal colloid, starch, albumin and others) disappeared on alkalisation and appeared on acidification. This is one of the convincing proofs that colloid aggregation can change at different reactions. It, moreover, strongly suggests that the decrease of the osmotic pressure of colloidal solutions under the influence of electrolytes may be most conveniently ascribed to a similar phenomenon.

So far as the results of our experiments were concerned the effects of three salts on the three colloidal solutions showed no qualitative difference among themselves.

Briefly speaking I am inclined to think, with previous investigators, that the diminished osmotic pressure under the influence of salt solutions depends upon an increased aggregation of colloidal particles.

The effects of anaesthetics (chloroform, ether, alcohol) were tested in the

same way. The only difference of the method lay in the use of a flask, instead of a beaker, to prevent the evaporation of these anaesthetics, connection with the manometer tube being made by a side tube. The solution inside the membrane was 15 cc., outside about 500 cc. The concentrations of anaesthetics were not always the same. Saturated solutions of chloroform in distilled water, 10 % ether, and 5 % alcohol were the most concentrated solutions in the series of experiments in each case. Roaf and Alderson [1907] found that anaesthetics such as chloroform, ether and carbon dioxide detached no salts from blood serum, which differed thus from other tissues (red blood corpuscles, brain, liver, muscle, kidney). Moore and Roaf [1907] observed that the presence of anaesthetics (chloroform) or organic solvents (benzene) did not alter the osmotic pressure of the serum protein (pig's serum). In their experiments the serum saturated with chloroform was placed against saline (0·175 %) also saturated with chloroform.

Despite the imperfections of the present osmotic pressure determinations the results of experiments with anaesthetics seem to favour somewhat positive effects though they are not very decisive.

Summary.

1. Salt solutions (SO_4Cu, Cl_2Ca, PO_4HNa_2) added to colloidal solutions (gum arabic, egg albumin, blood serum) decreased the osmotic pressure exerted by the latter. No qualitative difference was confirmed among the salts or among the colloidal solutions within the concentrations here employed. The remarkable reversibility of the osmotic pressure suggests, at once, that it is a physical phenomenon, *i.e.* increased aggregation of colloidal particles by the electrolytes.

2. The effects of anaesthetics (chloroform, ether, alcohol) on the osmotic pressure of colloidal solutions were studied. The results, although not quite definite, seem to show that there is some effect.

I wish to express my great indebtedness for Prof. B. Moore's constant direction in this work.

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XXXV. FURTHER OBSERVATIONS ON THE NATURE OF THE REDUCING SUBSTANCE IN HUMAN BLOOD.

By EVELYN ASHLEY COOPER and HILDA WALKER.

From the University of Birmingham.

(*Received April 3rd, 1922.*)

In the previous communication [1921] it was shown that the reducing power of human blood was sometimes increased by acid hydrolysis. This pointed to the presence in addition to glucose of a more complex substance, which was shown not to be glycogen, and thus appeared to be a disaccharide. The quantitative determination of this accessory substance was found to be complicated by two factors:

(1) the destructive effect of the HCl used for hydrolysis upon the reducing substance;

(2) the inhibitory effect of NaCl (formed by neutralisation after hydrolysis) upon the reduction of the copper carbonate by sugar.

Further work on the subject has since been carried out, and the results are recorded in the present paper.

First of all, it was necessary to select an acid, which did not itself destroy sugar, and the salts of which had no effect on the reducing process.

In the previous work sodium sulphate had been found to have no inhibitory effect, but the use of sulphuric acid for hydrolysis is inadmissible, because sodium sulphate is used for removing the proteins from the blood, and its presence would diminish the ionisation of the acid.

Further experiments showed that potassium chloride, like sodium chloride, had a retarding action on the reducing process, while the sulphate had no effect. The use of other chlorides for comparative purposes was not possible for various reasons. For example, ammonium chloride is decomposed by the alkali in the copper solution, and the liberated ammonia destroys the glucose, while the salts of several metals, *e.g.* Ca, Ba, Mg, give precipitates with the copper carbonate. The results, however, show that the chloride ion has a specific retarding effect.

Experiments with varying concentrations of sodium and potassium chloride showed that the retarding action first appeared when the concentration was approximately $N/1$. This inhibitory effect was found to be irreversible, *i.e.* when a solution of glucose containing NaCl was diluted with

a salt-free sugar solution, the reducing action of the sugar upon the copper solution was still (proportionately) retarded. The reduction was also retarded when the salt was added to the boiling mixture of sugar and copper solutions. This suggests that the compounds of glucose with the chlorides are much more stable than is commonly supposed. Bromides and iodides of the alkali metals also inhibited the reducing process.

Sodium citrate was moreover also found to exert an inhibitory action when present in concentration equivalent to 2 % citric acid, which is employed for hydrolysis in sugar analysis.

Di-sodium phosphate, on the other hand, had no inhibitory effect, and it thus seemed that phosphoric acid might be a suitable acid for hydrolysing the blood-sugar extracts. Separate portions of an extract (prepared by MacLean's method, as in the previous communication) were hydrolysed with $N/10$ HCl and $N/10$ H_3PO_4, and it was found that the reducing substance was destroyed to the extent of about 10 % by both acids.

Pure glucose was next dissolved in sodium sulphate solution, or in blood-extract, and the mixture hydrolysed with $N/10$ or $N/100$ HCl. The results obtained were similar to those obtained with blood-extract itself [Cooper and Walker, 1921]; sometimes there was no destruction of reducing substance at all, but occasionally a destruction occurred amounting to about 10 %.

These results afford an explanation of our previous work. Evidently the hydrolysable substance, which may be a disaccharide, is only occasionally present in blood, so that on hydrolysis there is often no increase in the reducing power of the blood-extract, or there may actually be a loss in reducing power, owing to destruction of some of the glucose. In fact, out of 18 samples of normal human blood examined, only six cases showed an increased reducing power after acid hydrolysis, and as a general rule the chief reducing substance present is glucose.

Two blood-extracts were subjected to dialysis. In one case the hydrolysable substance dialysed completely. In the other case, however, it was only partially dialysable, and this suggests that occasionally substances of intermediate complexity may be present in blood, possibly related to malto-dextrins.

The estimation of blood-sugar.

Up to the present time nearly all the methods for estimating sugar in physiological work are modifications of Fehling's method. Although fairly reliable, this is an empirical method, and the interaction of sugar with alkali copper solution is of great complexity. Recently, the iodometric method for estimating sugars has come to the forefront [see Baker and Hulton, 1920]. This is a simple method, consisting in the quantitative oxidation of aldoses to the corresponding carboxylic acid by means of iodine in alkaline solution, and it can be carried out in a few minutes at room temperatures. We have therefore attempted to adapt it to physiological work.

We have found, however, that in estimating pure glucose in concentration approximating to the average content of the blood-sugar extracts by the iodine method, slightly low results are obtained. Varying the proportion of alkali, and extending the period of reaction did not affect the results. In estimating the sugar in blood by this process, however, the results were twice as high as those obtained by MacLean's method. The alkali copper reagent is thus more selective than iodine in its action, and the iodometric method does not appear to be suitable for physiological work.

Influence of fatigue on the blood-sugar concentration.

Estimations of the blood-sugar by MacLean's method were carried out on normal persons immediately before, and directly after exercise. The persons examined were students, both men and women, of ages ranging from about 18 years to 26. Estimations were made on one man before and after half-an-hour of strenuous boxing, the other estimations on men were made before and after Rugby football, and on the women, before and after hockey. Of twelve experiments, there was a rise in the blood-sugar concentration in nine cases. The increase, as shown in the appended table, varied from 10 to 50 %, but in one case the amount was nearly trebled after $1\frac{1}{4}$ hours' play.

		% before	% after
Boxing. $\frac{1}{2}$ hour.	*A*	0·04	0·05
Rugby. 1 hr. 10 mins.	*B* (1)	0·052	0·077
	(2)	0·072	0·130
	C	0·097	0·090
	D	0·093	0·10
	E	0·115	0·10
	F	0·087	0·094
	G	0·118	0·094
Hockey. 1 hr. 15 mins.	*H*	0·087	0·253
	I	0·065	0·075
	J	0·118	0·150
	K	0·083	0·092

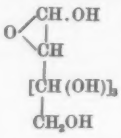
The player *B* is a first class forward, and in good training; he is of a very nervous and imaginative temperament, and before the match is always in a state of suppressed excitement. *C* and *D* are also fast players; *C* is of a quiet type, not easily roused to excitement. *E*, *F* and *G* were not in training, and did not take the game so seriously as the others.

Of the hockey players, *H* was playing back; she is of a highly nervous temperament, but well controlled. *I* and *K* were half-backs, and *J* played forward.

It will be seen that the players most easily roused to excitement over the game (*B* and *H*) are those whose blood-sugar has increased the most, although *H*, playing back, probably underwent less physical fatigue than most of the others. Psychic influences, therefore, probably play a considerable part.

Condition of the sugar in the blood.

We next considered the question of the structural condition of the sugar in the blood, and its bearing on physiological and pathological problems. It is well known that sugars do not merely exist as aldoses and ketoses, but may also pass into cyclic forms known as oxides. The ethylene oxide form:



is characterised by being extremely chemically reactive, and it decolorises permanganate rapidly.

Hewitt and Pryde [1920] showed that this active form of sugar could be actually formed by contact of an aqueous solution of glucose with the intestinal wall *in vivo*.

It is possible that in the animal organism sugar is normally metabolised in this active condition, and that an enzyme exists for transforming ordinary sugar as ingested into this form. Now it is known that fructose is more readily converted into the ethylene oxide structure than glucose, and that in diabetes the organism may still be able to metabolise fructose, although it has lost the power to deal with glucose. This suggests that the causation of diabetes is associated with some disturbance in the enzyme mechanism, which normally converts inactive sugar into the reactive ethylene oxide form.

We therefore proceeded to ascertain whether normal human blood can cause ordinary sugar to pass into this reactive state. A few cc. of fresh blood were placed in a small dialyser, immersed in ½–1 % solutions of glucose and fructose. At varying intervals of time, samples of the dialysate were withdrawn and examined, either polarimetrically or with a solution of permanganate. No evidence, however, was obtained that blood, under the above conditions, can produce the ethylene oxide form from ordinary glucose or fructose.

Since these experiments were carried out, Hewitt and Souza [1922] have found that even *in vivo* normal blood is unable to induce this structural change.

Summary.

1. Chlorides, bromides, iodides and citrates inhibit the reduction of copper carbonate by glucose, as carried out by MacLean's method. Sulphates and phosphates have no effect.

2. Glucose is slightly destroyed by boiling with $N/10$ HCl and $N/10$ H_3PO_4. The reducing substance present in blood is also destroyed by acid to about the same extent. This affects the determination of the hydrolysable reducing substances in blood.

3. Glucose is the chief sugar occurring in human blood, and reducing substances of a more complex nature are only occasionally present.

4. The estimation of blood-sugar by the iodine method gives results much higher than those obtained by MacLean's process, and the iodine method does not seem suitable for physiological work.

5. The blood-sugar concentration may rise considerably as the result of muscular exertion.

6. There is no evidence that human blood can transform ordinary glucose or fructose into the reactive ethylene oxide form.

7. A theory as to the causation of diabetes is put forward.

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XXXVI. BLOOD ENZYMES. II. THE INFLUENCE OF TEMPERATURE ON THE ACTION OF THE MALTASE OF DOG'S SERUM.

By ARTHUR COMPTON.

From Laboratoire de Chimie Biologique, Institut Pasteur, Paris.

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In a recent communication [Compton, 1921, 2], we have called attention to the fact that it is possible to divide mammals into two groups, depending upon whether the enzyme maltase is present, or not, in their blood-stream. For convenience, these groups have been named the "dog group" and the "rabbit group." The former group is represented in our experiments by such mammals as the dog, the pig, the ox, the horse, the goat, the sheep and the rat; while the latter group is represented by the rabbit, the guinea-pig, the cat and man.

In view of the general interest of the question, and the fact that it offers a starting-point for various researches, we have set out in the present investigation to determine, under certain well-defined conditions, the effect of heat on the enzyme as met with in the blood of the dog—an animal typical of the maltase group.

Experimental.

For this investigation three dogs were utilised (2-*P*, 3-*P*, 4-*P*). Blood was withdrawn aseptically by venous puncture from the jugular, the animals having been in general in the fasting state for 24 hours previously[1]. Dog 2-*P* was examined three times, an interval of three weeks elapsing between the examinations (1) and (2), and one week between the examinations (2) and (3); dog 3-*P* twice, 18 days elapsing between the examinations; while blood was only once examined from dog 4-*P*.

As soon as withdrawn, the blood was transferred to a sterile centrifuge tube, centrifuged, and the supernatant serum pipetted off from the clot. The serum thus collected was conserved in presence of a few drops of toluene in a closed test-tube, and utilised as required in the studies which follow.

I. *Activity in maltase of various specimens of dog's serum.*

The activity in maltase of the different specimens of serum was determined at the temperature of 47°, in an action of 16 hours' duration and concentration

[1] I desire to express here my indebtedness and thanks to Monsieur A. Frouin, for kindly supplying me with specimens of dog's blood for these experiments.

in maltose $M/20$: that temperature having previously been found under like circumstances to be the optimum temperature of a specimen of vegetable maltase prepared from *Aspergillus oryzae* [Compton, 1914].

For the determination, 90 mg. of pure hydrated maltose were weighed out into each of four clean test-tubes, to which were added 2 cc. of pure water (redistilled) to make a solution; then doses of the serum under investigation were added to the tubes as follows: 0·1, 0·2, 0·4, 0·8 cc. The contents of the tubes were then completed rapidly to 5 cc. with a further addition of water respectively as follows: 2·9, 2·8, 2·6 and 2·2 cc. Next, three drops of toluene were added to each tube, the latter shaken, closed with clean corks, and plunged into a water-thermostat regulated at or about 47°. After 16 hours' incubation, the tubes were withdrawn, the corks removed and the tubes heated for seven minutes in boiling water to stop the enzyme action. When cold, the contents of each tube were diluted to 50 cc., and 20 cc. of the diluted mixture employed to determine, by Bertrand's method, the proportion of maltose hydrolysed. The numbers obtained are set out in Table I.

Table I.

Lab. No. of the animal	Dose of serum employed cc.	Temperature of experiment ° C.	Percentage of maltose hydrolysed	Information as to the age of serum at the time of testing
2-*P* (1)	0·05	47·0–46·8	25·4	Fresh
	0·1		39·1	
	0·2		50·9	
	0·4		63·5	
	0·8		78·5	
2-*P* (2)	0·1	47·4–46·8	34·3	Two days
	0·2		46·4	
	0·4		61·9	
	0·8		71·7	
2-*P* (3)	0·1	47·5–46·0	31·2	Fresh
	0·2		49·4	
	0·4		60·3	
	0·8		71·7	
3-*P* (1)	0·1	47·0–46·9	18·3	Two days
	0·2		32·8	
	0·4		49·4	
	0·8		58·7	
3-*P* (2)	0·1	46·8	26·8	Fresh
	0·2		46·4	
	0·4		49·4	
	0·8		70·0	
4-*P*	0·1	47·2	47·9	One day
	0·2		68·4	
	0·4		78·5	
	0·8		80·3	

On plotting the percentage of maltose hydrolysed against the quantities of serum in action, these numbers give the curves indicated in Fig. 1.

An outstanding feature of this figure is the close relationship existing between the individual examinations of a given animal. Thus, we find dog 2-*P* giving a series of curves: 2-*P* (1), 2-*P* (2) and 2-*P* (3), crowded together in the same region of the figure. Indeed, the curves 2-*P* (2) and 2-*P* (3) are practically identical. Again, the same is true, although to a lesser degree, of

the dog 3-*P*: the curves 3-*P* (1) and 3-*P* (2) presenting a very similar allure, and being situated lowest in the diagram. The animal 4-*P*, on the other hand, represented by only a single examination, gives a curve which stands well apart, in a quite different region from the curves of the other dogs, being situated highest.

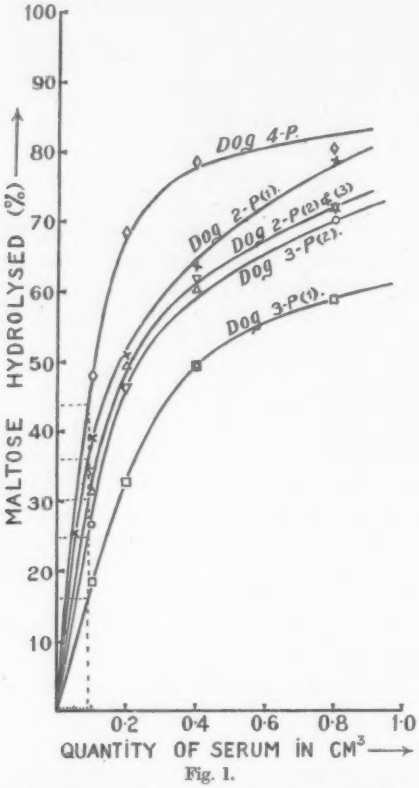

Fig. 1.

The conclusion therefore, would seem justified, that while the content of the blood serum of the dog in the enzyme maltase may vary considerably from animal to animal, still for the individual it is remarkably constant, varying only between comparatively narrow limits.

II. *Optimum temperature of the maltase of dog's serum.*

For reasons relating to another investigation, the quantity of serum utilised for the determinations of optimum temperature described in this paper is 0·09 cc. To employ conveniently this dose we operated as follows: 1·35 cc. of serum were measured out into a small 15 cc. measuring flask and the content made up to the 15 cc. mark with pure water. After shaking,

the mixture was distributed in portions of 1 cc. to a series of seven or eight test-tubes which had been prepared in advance, containing 90 mg. of maltose dissolved in 4 cc. of water and five drops of toluene, each tube being already placed in a water-thermostat regulated to a constant temperature. The object of this latter procedure was that the substrate solution in the tubes should already be in temperature equilibrium with the thermostat at the moment of adding the enzyme solution. The tubes were closed with clean sterile corks and incubated for 16 hours, after which the corks were removed, and the enzyme action stopped as previously. The proportion of maltose hydrolysed in each tube was determined as already described. The numbers obtained are set forth in Table II.

Table II.

Temperature at beginning and end of each experiment ° C.	Maltose hydrolysed %					
	Dog 2-*P*			Dog 3-*P*		Dog 4-*P*
	(1)	(2)	(3)	(1)	(2)	
30·3–29·1	—	—	—	—	—	12·8
36·8–37·2	—	—	—	—	—	24·0
37·0–37·1	—	12·8	—	—	12·8	—
37·3–37·0	—	—	—	10·0	—	—
37·5	19·7	—	—	—	—	—
41·9–42·0	—	—	15·5	—	24·0	—
44·5–45·2	26·8	—	—	—	—	—
45·8–46·0	—	—	—	—	—	46·4
46·1–46·0	—	—	—	24·0	—	—
46·8	—	—	—	—	—	43·5
46·9–46·8	—	25·4	—	—	—	—
46·9	—	—	—	—	34·3	—
47·0–46·8	—	—	—	24·0	—	—
47·0–47·2	31·2	—	—	—	—	—
47·2	—	—	21·1	—	—	—
49·6	34·3	—	—	—	—	—
49·8	—	—	—	—	—	52·5
49·8–49·9	—	28·2	—	—	—	—
49·8–50·0	—	—	—	—	40·6	—
50·0–49·5	—	—	—	28·2	—	—
54·5	43·5	—	—	—	—	—
54·5–54·8	—	—	—	32·8	—	—
54·6–54·9	—	—	—	—	45·0	—
54·8–55·0	—	—	31·2	—	—	63·5
55·0	—	37·5	—	—	—	—
57·5	—	—	—	—	43·5	—
58·3–57·6	—	29·7	—	—	—	—
58·0–58·5	31·2	—	—	—	—	—
58·1–58·5	—	—	—	—	—	46·4
58·2–58·0	—	—	—	28·2	—	—
58·4–58·3	—	—	25·4	—	—	—
60·0–60·1	—	28·2	—	—	—	—
61·0–62·0	11·4	—	—	—	—	—
61·5–61·8	—	—	—	—	24·0	—
61 8–62·0	—	—	—	12·8	—	37·5

On plotting the percentage of maltose hydrolysed against the mean temperature of the experiment, these numbers give the series of optimum temperature curves indicated in Fig. 2.

Fig. 2 reveals for the three animals studied the same optimum temperature: 55°, in an action of 16 hours' duration and reaction of medium that of the

serum diluted in pure water. It is of interest that this same optimum temperature is found, whether successive studies are made at different times of the same animal, or whether different animals are studied.

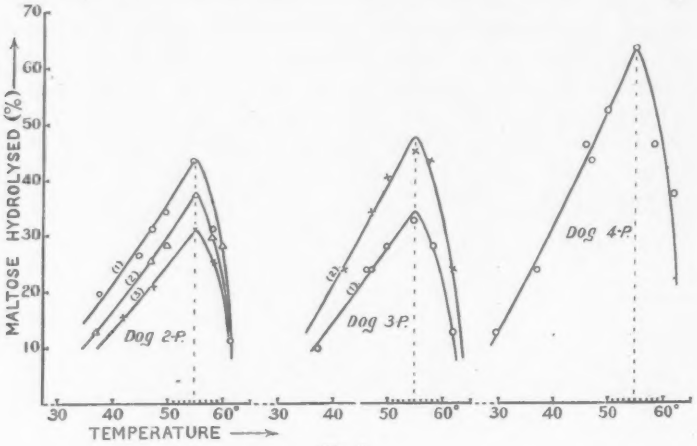

Fig. 2.

Conclusion.

The enzyme maltase, met with in the blood of the dog, exhibits from animal to animal a constant optimum temperature. This stability of optimum temperature constitutes an experimental fact which cannot but prove to be of considerable practical value as a reference point, in connection with any subsequent study of the enzyme. It is in contrast with the variability which characterises the actual amount of enzyme present at any time in the blood of different animals.

From what we know of the remarkable sensitiveness of the optimum temperature of an enzyme to variations of p_H of the medium [Compton, 1915; 1921, 1], there can be little doubt that the theoretical interpretation of the above effect lies in a certain constancy of the chemical reaction, or hydrogen-ion concentration, of the animal's blood serum. The same delicate mechanism which maintains constancy of the one, will obviously maintain constancy of the other.

From the point of view of general physiology, this stability of optimum temperature of a blood enzyme raises problems of even wider interest. For instance, one may ask how far it may be possible to define a species by humoral reactions of enzymic nature. That is a question to which we hope to return in the course of subsequent studies in this series.

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XXXVII. THE MODE OF OXIDATION OF FATTY ACIDS WITH BRANCHED CHAINS. II. THE FATE IN THE BODY OF HYDRATROPIC, TROPIC, ATROLACTIC AND ATROPIC ACIDS TOGETHER WITH PHENYLACETALDEHYDE.

By HERBERT DAVENPORT KAY and HENRY STANLEY RAPER.

From the Department of Physiology and Biochemistry, the University of Leeds.

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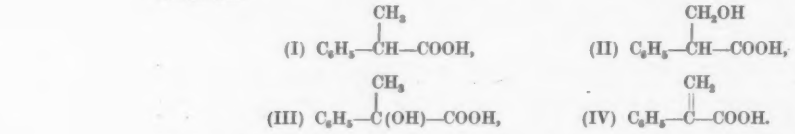
In a previous communication, Raper [1914] advanced an hypothesis to explain the mode of oxidation in the body of fatty acids substituted at the α-carbon atom by a methyl group. The experimental evidence available at that time indicated that these acids underwent oxidation in the same way as the straight chain fatty acids from which they were derived. It was suggested that this behaviour might be accounted for if oxidation of the α-methyl group took place first, giving rise to a derivative of the semi-aldehyde of malonic acid, which being very unstable would lose carbon dioxide to produce a normal fatty aldehyde. This aldehyde, in turn, would give rise to its corresponding fatty acid by further oxidation. The series of reactions would thus be as follows:

$$R{-}\underset{|}{\overset{CH_3}{CH}}{-}COOH \longrightarrow R{-}\underset{|}{\overset{CHO}{CH}}{-}COOH \longrightarrow R{-}CH_2{-}CHO + CO_2 \longrightarrow R{-}CH_2{-}COOH.$$

In this way an α-methylated acid would give rise to a fatty acid with a straight chain structure but containing one carbon atom less. In support of this hypothesis it was shown that isobutyric acid and α-methylbutyric acid, on oxidation with hydrogen peroxide, yielded propaldehyde and normal butaldehyde respectively.

Owing to the relative scarcity of data concerning the fate of branched chain fatty acids in the animal organism it was decided to investigate the fate of phenyl derivatives of the α-methylated fatty acids. The present communication is concerned with the simplest member of this series, namely, hydratropic acid (α-phenylpropionic acid), and some of its derivatives. Experiments with other members of the α-substituted series are in progress and will be communicated in due course. The results have been disappointing so far as the isolation of intermediate products of oxidation is concerned but the fate of the various acids investigated, (I) to (IV), has enabled conclusions to

be drawn which indicate the probable manner in which hydratropic acid is oxidised.



(I) $C_6H_5—CH(CH_3)—COOH$, (II) $C_6H_5—CH(CH_2OH)—COOH$,

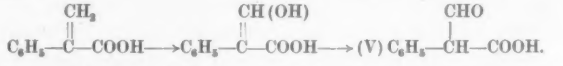
(III) $C_6H_5—C(CH_3)(OH)—COOH$, (IV) $C_6H_5—C(=CH_2)—COOH$.

Hydratropic acid (I) administered to dogs produced slight toxic effects in doses of 0·25 g. per kilo and was oxidised to the extent of about two-thirds. The remaining third was excreted in the urine, partly combined with glycine, and was dextrorotatory. A resolution of hydratropic acid, not yet complete, has shown that this was not the pure dextro form but a mixture of the dextro and laevo modifications in which the dextro component was in excess. No intermediate products of oxidation were detected. Inactive tropic acid (II) was found to be very resistant to oxidation, over 90 % of the acid given being recovered from the urine. The acid excreted, as expected, was optically inactive. Inactive atrolactic acid (III) was also very resistant to oxidation and over 80 % of this acid was excreted unchanged. It also was optically inactive. Atropic acid (IV), on the other hand, was found to be the most toxic of the four acids investigated, but in doses of 0·13 g. per kilo, which were tolerated, it was completely oxidised. No intermediate products were detected in the urine but a small but definite trace of succinic acid was excreted on two occasions and was not found on careful examination of the normal urine of the same dog. It is difficult however to connect this directly with the oxidation of atropic acid.

It is of great general interest to find that tropic acid is much more resistant to oxidation in the body than hydratropic acid, a result which the greater *in vitro* oxidisability of the hydroxylated acids (when acted on by the usual oxidising agents) would hardly lead one to expect. Dakin [1909] has observed the same phenomenon with β-phenylpropionic acid and β-phenyl-β-hydroxypropionic acid. The former is much more easily oxidised than the latter. These results show that the introduction of a hydroxyl group in the β-position in phenyl derivatives of propionic acid does not facilitate their oxidation in the body but rather hinders it. The fact that tropic and atrolactic acids are much less easily oxidised than hydratropic acid excludes them as possible intermediate products in its oxidation. It is also very improbable that hydratropic acid undergoes α-oxidation since either atrolactic acid, phenylglyoxylic acid, mandelic acid or benzoic acid would be expected as intermediate products. A careful search failed to reveal any of these, so that it may be assumed that hydratropic acid is oxidised in the β-position. Further, since the methyl group containing the β-carbon atom is unsubstituted both in atrolactic and hydratropic acids, it would be expected, that the former would be oxidised to a greater extent than was found to be the case. This makes it probable that some other factor besides the non-substitution of the

methyl group is important in facilitating the oxidation of hydratropic acid. We believe this factor to be the ability to form an unsaturated linkage between the α- and β-carbon atoms by the loss of two atoms of hydrogen. This is not possible in atrolactic acid but it is in hydratropic acid and would result in the formation of atropic acid. Atropic acid is not resistant to oxidation and although it may be urged that it is more toxic than hydratropic acid and therefore is not a likely intermediate product in the oxidation of the latter, it has to be borne in mind that the toxicity of a substance when produced slowly by metabolic processes in the tissues may be much less marked than when a comparatively large dose is introduced quickly by subcutaneous injection. Hydratropic acid, also, is distinctly more toxic than atrolactic and tropic acids. In the first case, doses of 0·25 g. per kilo give toxic effects whereas 1 g. per kilo of either of the other two acids gives no toxic symptoms. For these reasons therefore we are led to believe that atropic acid is the most likely primary oxidation product of hydratropic acid. It follows from this that the change from hydratropic acid to atropic acid is brought about by the direct oxidative removal of two hydrogen atoms and not by the loss of water from either tropic or atrolactic acids, since these are apparently not capable of conversion to atropic acid in the body. If they were they would be oxidised to a greater extent than was found to be the case. It is also possible that atropic acid may be produced from hydratropic acid by a process of dehydrogenation as pictured by Wieland [1912].

As to the further stages in the oxidation of hydratropic and atropic acids we can only speculate. From analogy with cinnamic acid β-oxidation might be expected with the formation of formylphenylacetic acid (V):



This would be unstable and lose carbon dioxide to give phenylacetaldehyde, or by further oxidation it might give phenylmalonic acid. The fate of the latter substance in the body is not known, but Dakin [1909] has described experiments in which phenylacetaldehyde in dilute alcoholic solution was given subcutaneously to dogs. About 84 % was completely oxidised and the remaining 16 % converted into phenylacetic acid which was excreted in the urine as phenaceturic acid. We have repeated and confirmed Dakin's experiment and found that by oral administration also a considerable part of the phenylacetaldehyde administered undergoes complete oxidation. Since neither hydratropic nor atropic acid gave rise to the excretion of phenylacetic acid it is not certain that phenylacetaldehyde represents an intermediate stage in their oxidation, although it would be expected to be such if the original hypothesis put forward previously [Raper, 1914] were correct. It is however not improbable that phenylacetaldehyde produced *in vivo* may undergo complete oxidation in spite of the fact that when introduced orally or subcutaneously it is partially oxidised to phenylacetic acid.

In a recent communication, Hanke and Koessler [1922] have called attention to the phenomena of the oxidation of fatty acids in the body in relation to their electronic structure. The arguments put forward in the case of butyric acid, for which the following electronic structure is developed, will serve as an example:

$$\underset{\gamma}{-\overset{-}{C}-} \; \underset{\beta}{+\overset{-}{C}+} \; \underset{\alpha}{-\overset{-}{C}-} \; \underset{+}{+\overset{+}{C}+}$$

In this formula the α- and γ-carbon atoms are represented as quadruply negative and the carboxylic carbon atom as quadruply positive, whereas the β-carbon atom is doubly positive. Evidence is brought forward by Hanke and Koessler which indicates that quadruply positive or negative carbon, and especially the former, is resistant to oxidation. This being so, it would be expected that butyric acid when oxidised would be attacked most readily at the β-carbon atom which is only doubly positive. It is therefore suggested that in this way the predominance of β-oxidation of fatty acids in the body may be explained. On our part, we feel that there is, as yet, insufficient knowledge, either qualitative or quantitative, and especially the latter, of the mechanism of oxidation processes on which an explanation in relation to their electronic structure can be based. The idea that β-oxidation is predominant is supported essentially by qualitative evidence for there is little of a quantitative nature. There are no animal experiments on record which enable one to postulate that butyric acid is oxidised only or chiefly in the β-position. Further, Cahen and Hurtley [1917] have shown that when butyric acid is oxidised with hydrogen peroxide, a process which simulates to some extent oxidation in the body, the main product is succinic acid and not acetone, so that oxidation in this case is most marked at the γ-carbon atom. There is also evidence which indicates that in certain cases, β-oxidation, when it occurs, may be preceded by "desaturation" between the α- and β-carbon atoms. The oxidation of hydratropic acid to produce atropic acid, as suggested above, is a case in point, but others are: the production of cinnamic acid from phenylpropionic acid [Dakin, 1909] and the formation of furfurylacrylic acid from furfurylpropionic acid [Sasaki, 1910; Friedmann, 1911]. Again, the electronic formulae of β-phenyl-β-hydroxypropionic acid and β-phenyl-propionic acid, developed by Hanke and Koessler, show that in both compounds the β-carbon atom is doubly positive and the α- and carboxylic-carbon atoms quadruply negative and quadruply positive respectively. If the ease of oxidation in the body of a particular carbon atom of a substance is sufficiently explained by the electronic formula then there would be no reason to expect that these two substances should not undergo oxidation equally readily. As a matter of fact the non-hydroxylated acid is much more readily oxidised. The experiments with hydratropic and tropic acids, described in the present communication, show equally marked differences and do not seem to be capable of adequate explanation on an electronic basis

alone. It would seem that until we have a more definite knowledge of the mechanism of the oxidation process, its explanation, based solely on views of polarity in the substance oxidised, or on its electronic structure, cannot be entirely successful. These views, developed largely by a study of the phenomena of addition and substitution in organic compounds, have given a satisfactory general explanation of previously inexplicable phenomena; but with the meagre quantitative data at our disposal and our lack of knowledge as to how oxidation of very simple substances takes place either inside or outside the body, the polarity alone of the atoms in the substance oxidised is insufficient as a basis of explanation. As an instance of the probable complexity of an apparently simple process such as the oxidation of carbon monoxide to carbon dioxide when the former is burnt in moist air, the work of von Wartenberg and Sieg [1920] may be referred to. It is suggested that in this oxidation four intermediate reactions are concerned. Firstly the production of formic acid by the addition of water, secondly its dehydrogenation to produce carbon dioxide and hydrogen, thirdly, oxidation of the hydrogen to produce hydrogen peroxide and lastly decomposition of the peroxide with the formation of water and oxygen. If the oxidation of carbon monoxide is as complicated as this scheme represents, then it is at least probable that the processes of oxidation of fatty acids are not less complex.

Experimental.

Methods. Unless stated otherwise, the acids used in this investigation were administered hypodermically as sodium salts in aqueous solution. The urine was collected from the time of the first dose until 36 hours after the last dose was given. Urines were preserved on ice. Volatile products were sought for by distilling all, or part of the urine in steam and examining the distillate. The urine was then concentrated under reduced pressure to about one-fifth of its original volume and acidified strongly with syrupy phosphoric acid. Subsequently it was extracted with ether in a continuous extractor for 30 to 48 hours. The ether extract was then examined for unchanged acid or products of oxidation.

Hydratropic acid. The acid was obtained in two ways. Firstly, by the reduction of atropic acid with sodium amalgam and secondly, by the oxidation of hydratropic aldehyde with silver oxide in the presence of a slight excess of sodium hydroxide. The hydratropic aldehyde was prepared by the method of Tiffeneau [1907]. The former method was found to be the more expeditious. The acid was purified by distillation under reduced pressure and had B.P. 148–9° (14 mm.). In doses of 0·25 g. per kilo a slight lassitude was developed but no other ill effects were noted. Dogs, only, were used in this experiment and larger doses were not tried. No volatile products of oxidation were detected when the urine was distilled with steam. The ether extract from the acidified urine was obtained as a light brown syrup which refused to crystallise. Extraction with warm light petroleum removed some unchanged hydratropic

acid and traces of fatty acids. The latter were removed as insoluble barium salts and the hydratropic acid recovered was weighed. In this way, in two experiments, 3 and 22 % of the acid originally given was recovered. Its equivalent was 151 (calculated 150), and it was dextrorotatory. In absolute alcohol (concentration 3 %), it had $[\alpha]_D^{20}$ + 21·2° to + 26·9°. The ether extract, after removal of the hydratropic acid as just outlined, was treated in many ways to induce it to crystallise but all were fruitless. It was dextrorotatory, reduced Fehling's solution on boiling and gave the orcinol reaction, indicating the presence of a glycuronic acid derivative. The original urine also gave these reactions. In addition, the ether extract contained some nitrogenous substance. Having failed to obtain any crystalline compound from this extract, and suspecting the presence of a glycine derivative because of the marked nitrogen content, it was hydrolysed by boiling with concentrated hydrochloric acid for an hour. After hydrolysis, the solution was diluted and extracted with light petroleum and then with ether. The latter yielded only a trace of oily substance which was neglected, but the former was found to contain dextrorotatory hydratropic acid (equivalent 150·8). In 3 % solution in absolute alcohol it gave $[\alpha]_D^{20}$ + 27·2°. The hydrolysate after ether extraction was evaporated to small bulk under reduced pressure and esterified with absolute alcohol saturated with hydrochloric acid. It was seeded with a crystal of glycine ester hydrochloride and placed on ice for two days, whereby a mass of crystals of glycine ester hydrochloride was obtained. The identity of the glycine was confirmed by benzoylation, when hippuric acid, M.P. 186–7°, was obtained. Careful search failed to reveal any other definite products of hydrolysis, phenylacetic, tropic and atropic acids being specially sought for. The chief substance present in the ether extract of the urine thus appeared to be hydratropylglycine. A sample of this substance was made by the Schotten-Baumann method from hydratropyl chloride and glycine and was found to be more difficult to crystallise than the commoner acyl derivatives of glycine such as phenaceturic and hippuric acids, and its contamination with some oily impurity, possibly a glycuronate of hydratropic acid, with about the same solubilities, explains its non-crystallisation from the ether extract. In a later experiment it was found possible to obtain crystalline hydratropylglycine from this extract with considerable loss, by the following method. The acids extracted from the urine by ether, after removal of unchanged hydratropic acid, were converted in turn into sodium salts and ferric salts. The acids obtained from the latter by decomposition with dilute sulphuric acid and extraction with ether were mixed with half their equivalent of sodium hydroxide and the free acids present in the solution shaken out with ether. The remaining sodium salts were now decomposed with dilute sulphuric acid and the liberated acid extracted with ether. This extract crystallised readily on inoculation with hydratropyl glycine and after two recrystallisations from water had M.P. 103°, which agreed with that of the synthetic product made from the dextrorotatory hydratropic acid recovered

from the urine, and glycine. The pure inactive glycine derivative has M.P. 105°. In one experiment carried out as quantitatively as possible for the estimation of the hydratropic acid excreted, either free or combined, three doses, each of 1·5 g., of the acid were given on three successive days. It was found that 0·156 g. (= 3·5 %) of the acid administered was present in the free state and 1·225 g. (= 27 %) in combination with glycine, or possibly partly with glycuronic acid. In order to determine whether any derivative was present in the urine as a glycuronate the concentrated urine after the usual ether extraction was freed from phosphoric acid by baryta and the reducing substance precipitated by basic lead acetate. This precipitate was decomposed with hydrogen sulphide and to the resulting solution, after removal of hydrogen sulphide in a current of air, dilute sulphuric acid was added to make the concentration about 5 %. It was boiled for eight hours and the resulting solution, after clarification with charcoal, extracted continuously with ether for eight hours. The ether extract contained only a trace of resinous substance. The reducing substance was probably, therefore, free glycuronic acid which had originally been combined with hydratropic acid and which had been split off during the ether extraction of the concentrated and strongly acidified urine. It is well known that many glycuronates are easily hydrolysed under such conditions.

Tropic acid. Inactive tropic acid was used in these experiments and its fate in both cats and dogs was determined. Doses of 1 g. per kilo caused no noticeable ill effects. The urine on steam distillation yielded no volatile oxidation products. The ether extract of the concentrated urine, on removal of the ether, gave a brown mass which crystallised at once on cooling and after one recrystallisation gave pure tropic acid, M.P. 118–119°. The acid was optically inactive. In two preliminary experiments, one on a cat and the other on a dog, the urine was extracted with ether for only 12 hours. In the first, 2·8 g. of the acid were given and 2·25 g. (80 %) of the pure acid were recovered from the urine. In the second, 5·9 g. of the acid yielded 4·4 g. (74 %) in the urine. A third experiment was carried out and the extraction with ether continued for 30 hours, when all the acid had been extracted. Of 2·94 g. of the acid administered, 2·77 g. were recovered, *i.e.* 94 %. Examination of the mother liquors after the separation of the tropic acid failed to reveal the presence of any oxidation products.

Atrolactic acid. The inactive acid was prepared from acetophenone by Spiegel's method using the modification described by McKenzie and Clough [1912]. Both dogs and cats were used for the experiments and doses of 0·9 g. per kilo were well tolerated, no ill effects being noted. No volatile oxidation products were detected in the steam distillate. The residue from the ether extract crystallised on cooling and was extracted with hot benzene to remove unchanged atrolactic acid. Most of the residue was soluble in benzene but a small amount of brown tarry substance remained after the extraction. This was dissolved in water and re-extracted with ether. The ether residue was

then repeatedly extracted with moist light petroleum whereby a further quantity of atrolactic acid was obtained. A small amount of brown oily substance remained which was insoluble in cold water, benzene or light petroleum and would not crystallise. The only definite substance obtained from the urine, therefore, was atrolactic acid. It was optically inactive and melted at 92–3° after one recrystallisation from water. In one experiment on a dog, 5·8 g. of the acid were administered and 4·9 g. (84 %) recovered from the urine. In another case two cats were used and of 4·5 g. of the acid administered, 3·42 g. (76 %) were recovered from the urine.

Atropic acid. The acid used in these experiments was prepared from tropic acid by boiling with 10 % aqueous baryta for 18 hours. It was obtained quite pure after one crystallisation from alcohol and had M.P. 106–7°. The yield was 67 % of the theoretical. Doses of 0·4 g. per kilo were invariably fatal both in cats and dogs within 30 hours and no oxidation products or unchanged acid were found in the urine. A dose of 0·2 g. per kilo, given to a dog, produced only slight lassitude, but when repeated after 24 hours caused death at the end of 60 hours. Doses of 0·13 g. per kilo were well tolerated. In one instance a dose of 0·18 g. per kilo, given to a dog, caused haematuria but the animal recovered. The urine on steam distillation yielded no detectable oxidation products. The residue of the ether extract was very small in amount. It was dissolved in hot water, clarified with charcoal, and on standing deposited a small amount of a colourless, crystalline substance. In one experiment in which 1·9 g. of the acid were given, 0·04 g. of this crystalline substance was obtained, and in another, 2·8 g. yielded 0·05 g. It was recrystallised from a mixture of nine parts benzene and one part alcohol and had M.P. 178–9°. On heating above its melting point it gave a crystalline sublimate. It was soluble in water and alcohol, slightly in ether and almost insoluble in chloroform and benzene. It gave insoluble ferric and lead salts but a soluble calcium salt. When recrystallised from dilute nitric acid it melted at 183°. The ammonium salt heated with zinc dust gave the pyrrole reaction. These properties agree with those of succinic acid but enough material was not obtained for analytical identification. No other substance was isolated from the urine. A control experiment with a 48 hours sample of urine from the same dog to which atropic acid had been given yielded none of the crystalline acid on ether extraction.

Phenylacetaldehyde. In a mixture of four parts alcohol to six parts water (by volume), 0·93 g. of phenylacetaldehyde was dissolved and given subcutaneously to a cat weighing 2 kilos. The cat appeared to be affected by the alcohol and was drowsy for two days, but recovered. The urine was collected for four days and on steam distillation yielded no detectable oxidation products. The ether extract was dissolved in water, distilled with steam to remove acids, again acidified with phosphoric acid and extracted with ether. The ether extract yielded a small amount of oily substance which crystallised after standing for two days. The mass was rubbed up with a little water and

filtered. In this way 0·26 g. of a crystalline acid was obtained. After recrystallisation from water it gave a very unsharp M.P., the main part melting at 135–140° and the rest at 170–180°. It appeared therefore to be phenaceturic acid mixed with some higher melting substance. By boiling with 20 % sulphuric acid for a short time the phenaceturic acid was hydrolysed and the resulting phenylacetic acid was extracted with benzene. It weighed 0·102 g. and was not quite pure. The higher melting substance was extracted by ether from the acid liquid and proved to be hippuric acid, which is more resistant to hydrolysis with hot dilute acids than phenaceturic acid. In this experiment therefore, not more than 10 % of the phenylacetaldehyde given appeared as phenaceturic acid. A second experiment was carried out on a dog to test the effect of oral administration, the use of alcohol as a solvent for subcutaneous injection being objectionable. 1·9 g. of phenylacetaldehyde in its own volume of alcohol were administered to a dog by the mouth in gelatin capsules. No abnormal symptoms were observed. The urine was collected for the ensuing 48 hours and was treated in the same way as that from the cat. A fairly pure crop of crystals of phenaceturic acid was obtained (M.P. 141–3°). It weighed 0·85 g. The mother liquors on hydrolysis yielded 0·1 g. of impure phenylacetic acid and again a little hippuric acid was isolated. The phenaceturic and phenylacetic acids thus obtained correspond to 0·62 g. of phenylacetaldehyde, which represents 32 % of that administered. Nearly 70 % had therefore been completely oxidised. These experiments thus confirm those of Dakin [1909].

Summary and Conclusions.

1. The experiments described in this paper were carried out to determine the mode of oxidation in the body of phenyl derivatives of fatty acids with a branched chain structure.

2. The acids investigated were, hydratropic, tropic, atrolactic and atropic acids. In addition previous observations by Dakin on the fate of phenylacetaldehyde were confirmed.

3. Hydratropic acid is moderately well oxidised and undergoes a partial resolution in the body, the unoxidised acid found in the urine being dextrorotatory. Atropic acid is also well oxidised and is more toxic than the other three acids. Tropic and atrolactic acids are comparatively resistant to oxidation.

4. From the observations made, it is concluded that hydratropic acid on oxidation is converted directly into atropic acid (though this was not directly proved), and that this then undergoes complete oxidation.

5. Phenylacetaldehyde largely undergoes complete oxidation in the body and may therefore be an intermediate product in the oxidation of hydratropic and tropic acids.

We desire to express our thanks to Messrs Boots Pure Drug Co. Ltd., for a gift of residues from the saponification of atropine. From these the tropic, atropic, and part of the hydratropic acids used in our experiments were obtained.

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XXXVIII. STRUCTURES IN ELASTIC GELS CAUSED BY THE FORMATION OF SEMIPERMEABLE MEMBRANES.

By EMIL HATSCHEK.

(*Received April 20th, 1922.*)

When a reaction is produced in a gel, the latter may undergo slight changes which, however, are not striking or even obvious, and have received little attention compared with that devoted to the characteristics of insoluble precipitates obtained in the gel. These changes are, generally speaking, alterations in the water content of the gel, such as dehydration by contact with very concentrated salt solutions, *e.g.* the 20 or 25 % silver nitrate solution used in the classical Liesegang experiment; or increased hydration caused by the diffusion of acid or of one of the strongly lyotropic salts such as iodide or thiocyanate. If the experiments are carried out in test-tubes, these changes show themselves most readily in alterations of the gel meniscus.

Much more complicated conditions arise, and the gel itself develops a complicated structure, when the reaction leads to the formation of a semipermeable membrane. The most convenient and striking instance is the formation of copper ferrocyanide in gelatin gel, and the most suitable arrangement is as follows: clean test-tubes are filled to about half their height with a gelatin sol containing 10 g. of air dry gelatin in 100 cc. of a 2 % solution of crystallised potassium ferrocyanide ($K_4Fe(CN)_6 . 3H_2O$). After setting completely—which is somewhat retarded by the ferrocyanide—the gel is covered with a 5 % solution of crystallised cupric chloride or cupric sulphate ($CuCl_2.2H_2O$ or $CuSO_4.5H_2O$ respectively), or, as will be explained below, with a mixed solution of cupric and sodium sulphate. The molar concentrations are accordingly $M/21$ $K_4Fe(CN)_6$ in the gel, and $M/3{\cdot}4$ $CuCl_2$ or $M/5$ $CuSO_4$ in aqueous solution. The molar concentration of the latter is thus considerably greater than the molar concentration of the salt in the gel, the necessary condition when a reaction producing an insoluble compound is to proceed into the gel.

A few minutes after putting on the copper salt solution a pale brown and perfectly transparent membrane of copper ferrocyanide can be noticed. The next change to be noticed is a gradual sinking of the surface of the gel. In the first stages there is no great alteration in the curvature of the meniscus, but it appears to travel down bodily, leaving a thin film of gel adhering to the glass. The constant formation or exposure of new surface entails fresh

formation of copper ferrocyanide, and the layer of gel on the glass as well as the meniscus turns dark brown and opaque. If the preparation is observed superficially only and at long intervals, this shifting of the meniscus and the formation of a dark brown zone on the glass seem to be all that happens. Careful observation however shows the process to be discontinuous: the curvature of the meniscus increases and finally the membrane composing it *bursts* somewhere near the centre, the copper salt solution penetrates through the crack and a fresh membrane is formed.

What happens can be readily deduced *a priori* from known properties of the semipermeable membrane and of elastic gels. As soon as the membrane is formed, water flows through it from ferrocyanide solution in the gel to the strongly hypertonic copper salt solution above it: in other words, the gel "dries." In doing so it behaves exactly as if drying proceeded in the usual way by evaporation into the atmosphere, *i.e.* the meniscus gradually sinks and becomes more and more curved. The surface film now consists of very concentrated gel and—provided the gelatin adheres well to the glass—is in tension. At the same time, owing to the withdrawal of water and the low diffusion velocity of the potassium ferrocyanide, with a molecular weight of 368·5, the concentration of the latter in the zone immediately below the membrane increases markedly; this can easily be seen by the colour, which is a clear yellow, although the rest of the gel, with the initial concentration of 2 %, is barely coloured. The ferrocyanide has, like the other cyanides, a marked softening effect on gelatin, which shows itself in concentrations of about 10 %, so that the layer below the tough surface film is considerably softer than the body of the gel. The surface film accordingly is torn off when the tension has increased sufficiently, and is at the same time ruptured by the atmospheric pressure, the copper salt solution penetrating under it. The whole process then repeats itself, and this continues until the copper salt solution, which is continually diluted, becomes approximately isotonic with the potassium ferrocyanide solution in the gel.

If the gelatin does not adhere to the glass wall sufficiently, it begins to contract below the reaction zone and copper salt solution flows into the space thus formed, covering the exposed gelatin surface with a semipermeable membrane. This leads to further shrinkage and finally a thin cylinder of very tough gel only may be left.

The analysis of the process, set forth above, was confirmed by a detailed examination of a large number of preparations. In the first instance the test-tubes were cut through about 1 cm. below the bottom of the reaction zone. Immediately the cylinder of gel is cut across, it contracts sharply and develops a deep constriction below the last membrane. The dehydrated gel containing the precipitate of copper ferrocyanide no longer adheres to the glass, and the reaction zone can, with a little care, be removed intact from the tube and cut through longitudinally. Two halves of such preparations, made in large boiling tubes 35 mm. in diameter, are shown in Fig. 1 (Plate IV).

An intact test-tube, in which the reaction has proceeded for some days, is illustrated in Fig. 2. The rupture in the last membrane but one is clearly visible, and the membrane in course of formation, which is still almost transparent, can be seen faintly. Fig. 3 shows a preparation in which the gel has detached itself from the glass and has become covered completely with copper ferrocyanide; the consequent shrinkage is very marked.

If the reaction proceeds throughout the gel, without the latter detaching itself, the final aspect of the preparation is extremely remarkable. It then consists of a thin tube of tough gel with a large number of septa, convex towards the lower end of the cylinder and always ruptured somewhere near the centre. A cross section through a specimen of this description is shown in Fig. 4. It may be mentioned that this preparation was obtained by using a mixed solution of copper and sodium sulphate, to which reference has been made, instead of copper sulphate alone. The function of the copper salt is a twofold one: it provides material for the formation of the semipermeable membrane, and it maintains the osmotic pressure so that dehydration of the gel takes place. The latter function can of course be performed equally well by a salt which does not enter into the reaction, and sodium sulphate has the advantage of not tanning the gelatin to the same extent as copper and other heavy metal sulphates. It is the latter action which seems to lead, or to contribute largely, to the detaching of the gel from the glass, which makes it difficult to obtain specimens showing more than two or three septa with copper salt alone. Incidentally the condition of the glass surface is of considerable importance: test-tubes cleaned carefully with bichromate-sulphuric acid mixture etc. consistently gave bad results, while new test-tubes simply dusted with a dry cloth gave the best.

If the explanation of the formation of septa, which has been given above, is correct, it follows that they should be formed in the course of any reaction which satisfies the following three conditions: (1) a semipermeable membrane is produced; (2) the solution above the gel is, and remains, hypertonic, and (3) the gel contains a lyotropic agent which, in moderately high concentration, reduces its modulus and tensile strength. Only one such combination—apart from the copper ferrocyanide reaction—readily suggests itself: gelatin gel containing potassium iodide as lyotropic agent, and on it a concentrated sugar solution containing tannin. The general results were the same as with copper ferrocyanide: depression of the meniscus, followed by the detachment and rupture of membranes. The latter were very irregular, probably because one set only of concentrations, chosen at random, was tried; a detailed investigation appeared unnecessary in view of the somewhat artificial nature of the arrangement.

The results described show that the possibilities of periodic structures in gels, as the result of continuous and uncontrolled diffusion, are by no means exhausted by the normal Liesegang phenomenon. In the latter the gel, while of course influencing the character of the results very considerably, primarily

serves as the support for periodic deposits of insoluble reaction product, and does not undergo any profound changes. If a semipermeable membrane is formed by the reaction, the gel itself becomes segmented and the final structure is one which it would be extremely difficult to explain *a posteriori*—quite as difficult as it was to explain other periodic structures before the study of the Liesegang rings provided a clue. While the author does not know any natural structure resembling those described and while he is, generally speaking, by no means inclined to overrate the cogency of the conclusions drawn from artificial imitations of natural forms, he ventures to think that the highly complicated consequences which follow the formation of a semipermeable membrane in a suitable elastic gel may have some biological or histological interest.

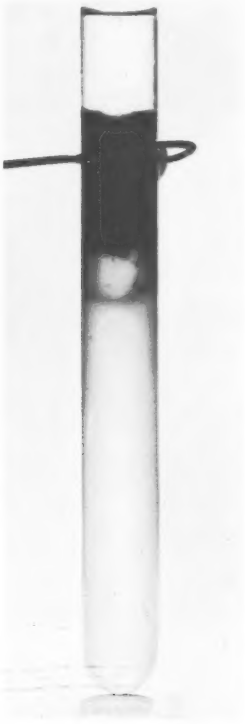

Fig. 2

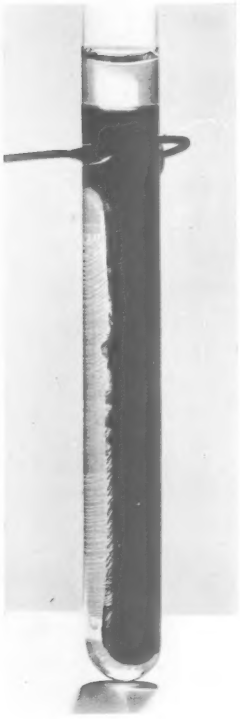

Fig. 3

Fig. 1

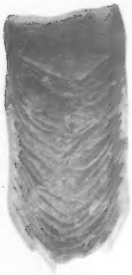

Fig. 4

XXXIX. A MODIFICATION OF BASAL DIET FOR RAT FEEDING EXPERIMENTS.

By MURIEL BOND.

From the Physiological Laboratory, London (Royal Free Hospital) School of Medicine for Women.

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Caseinogen, the usual protein constituent of a basal diet for rat feeding experiments, possesses several disadvantages. Owing to its natural association with milk fat it is difficult to render it free of fat-soluble *A*—if the purification be done by alcohol and ether extractions the cost of the material is greatly increased, and if the caseinogen is treated by the Drummond method [Coward and Drummond, 1921], careful supervision is required to ensure a proper oxygenation and a constant temperature. In addition it often seems impossible when using caseinogen to obtain a deficiency curve, although apparently no fat-soluble *A* is present in the diet. The rats continue to grow steadily and seem apparently healthy.

In view of these difficulties which were experienced while investigating the fat-soluble *A* content of certain foods, some rat feeding experiments were started, in which for the caseinogen dried egg white was substituted. Dried egg white, unlike caseinogen, contains very little associated fat and if it is an adequate protein when used in a percentage similar to that normally used for caseinogen, would be considerably cheaper.

The first series of experiments was done with a diet containing 30 % protein—instead of the usual 20 %—as analyses of egg white have shown it to be somewhat deficient in tyrosine.

When the diet was first used the dried egg white was crushed and the dry protein mixed with the other ingredients of the diet. It was found, however, that on this diet many of the rats developed diarrhoea and little or no growth was obtained. A diet was then tried in which the dried egg white was soaked overnight in tap water and the solution gently heated to coagulate the proteins. While heating the solution it was constantly stirred and the broken clot was then mixed with the other constituents. A fairly solid mass was thus obtained, which was readily taken by the rats and was satisfactorily digested[1].

The experiments were carried out on young healthy albino rats, weighing about 40–60 g. at the beginning of the experiments (see Fig. 1). Usually the

[1] Subsequently I read Bateman's paper [1916] on the digestibility of egg proteins and find that his results and mine are completely in accord.

young rats were fed on bread and milk for about a week previously and any animal not showing a decided gain in weight during that time was discarded. The rats were fed twice daily—usually at 9 a.m. and 3 p.m.— and were weighed twice a week. The amount of food given was so arranged that there was still a little left uneaten when a fresh quantity was due.

Each litter was divided into two groups. One group received diet *A* and the other group diet *B*.

	Diet *A*	Diet *B*
Soaked and cooked egg white	30 g.	30 g.
Salts (Hopkins' mixture) ...	5 g.	5 g.
Marmite	5 g.	5 g.
Strained lemon juice	5 cc.	5 cc.
Potato starch	40 g.	40 g.
Fat { butter	15 g.	—
Fat { hardened cotton seed oil	—	15 g.
Water to drink	*ad lib.*	*ad lib.*

The rats on the *B* diet showed a growth curve, which in a few weeks began to flatten out. These rats also frequently suffered from eye inflammations. The rats on the *A* diet grew well, appeared to be healthy and when they were mated reproduction was obtained (Fig. 1). In this series of experiments 20 rats were used.

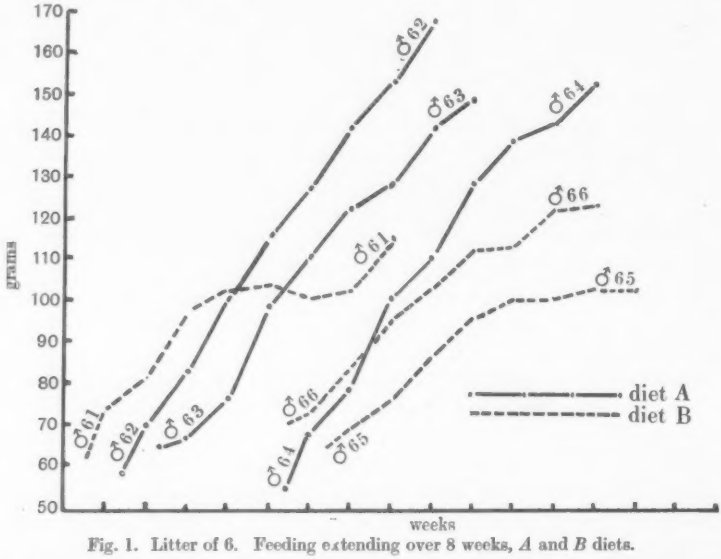

Fig. 1. Litter of 6. Feeding extending over 8 weeks, *A* and *B* diets.

The results of feeding the lactating rat and her growing young on the egg white diet are not very conclusive. One litter has been successfully reared on this diet, but as a rule it was found that the young died about the 20th–30th day after birth, and the mother became ill unless the diet was changed to one of bread and milk as soon as the young rats began to move about the cage.

This is probably due to the large proportion of protein in the diet [Hartwell, 1921, 1922]. Hartwell has shown 30–40 % protein to be excessive during lactation, although the experiments described by her are somewhat different from those described here.

1. In the experiments described in this paper the diet used for the lactating rats always contained all the necessary food factors and had been given to the mother rats during growth and gestation.

2. The egg white used has been soaked and cooked, whereas in the cases quoted by Hartwell, the protein was used crushed, dry and uncooked. This condition of the egg white in itself frequently leads to diarrhoea and death in non-lactating and non-sucking rats.

3. The unhealthy symptoms were not observed as early as in her experiments—the young rats always having begun to move about the cage and get food for themselves before they began to show signs of weakness. The characteristic spasms and screaming fits observed by Hartwell were never noticed, but the young rats became less active, were cold and died after a period of coma. Weighing of the young rats was not carried out so that it is not possible to say when growth ceased.

A second series of experiments is now in progress, in which the rats are receiving a diet containing only 20 % egg white. This is a diet comparable to that normally used, but with the substitution of egg white for caseinogen. The growth of rats on the 20 % diet is being compared with that of rats on the 30 % diet and so far no signs of inadequacy can be detected among the rats on the lower protein diet. There has not yet been time to ascertain whether this 20 % diet is adequate for reproduction nor whether it is more satisfactory than the 30 % diet during lactation.

A point in favour of egg white as a source of protein seems to be the constancy with which, when this material is used, the characteristic flattening of the growth curve is obtained if the diet be deficient in fat soluble *A*—a constancy not always obtained when caseinogen is used.

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XL. SYNTHESIS OF VITAMIN *A* BY A MARINE DIATOM (*NITZSCHIA CLOSTERIUM* W.Sm.) GROWING IN PURE CULTURE.

By HENRY LYSTER JAMESON, JACK CECIL DRUMMOND and KATHARINE HOPE COWARD (*Beit Memorial Research Fellow*).

From the Biochemical Laboratories, Institute of Physiology, University of London.

(*Received April 28th, 1922.*)

During the early stages of an investigation of the nutrition of certain species of marine molluscs it became necessary to gain some information concerning the food value of a number of marine plants upon which the shell-fish directly or indirectly feed[1].

Amongst other dietary factors to be studied that known as vitamin *A* claimed our early attention. By now it is generally understood that the higher land animals draw their supplies of this indispensable factor directly or otherwise from green plants, since their own tissues do not appear to possess the power to synthesise this organic complex. That a parallel condition would exist in the sea appeared to us to be highly probable from the fact that certain marine algae had been found to share with land plants the power to synthesise vitamin *A* [Coward and Drummond, 1921]. The algae examined in the former work were Fucus, Ulva, Cladophora, Polysiphonia and Enteromorpha, but these are not truly representative of the ultimate food supply of the species of molluscs with which we were working. We therefore decided to test whether a typical unicellular marine alga can synthesise vitamin *A*.

To obtain the necessary material it was decided to grow the organisms in pure culture following the technique described by Allen and Nelson [1910]. Pure cultures of three common marine diatoms, *Nitzschia closterium* W. Sm. *forma minutissima*, *Thalassiosira gravida*, and *Skeletonema* were very kindly placed at our disposal by Dr E. J. Allen, F.R.S., Director of the Marine Biological Laboratory, Plymouth.

To grow the relatively large quantities of the organisms required for the feeding tests was not a simple matter, and in the case of two of the three species named our attempts failed owing to their slow rate of growth. With Nitzschia, however, we found it a relatively simple matter to prepare ample

[1] Unfortunately owing to the sudden and untimely death of our colleague Dr H. Lyster Jameson the main line of this work has been discontinued. The present communication records the chief result which we had obtained up to the time of his death, by which science and particularly marine biology suffers a grievous loss. J. C. D.

quantities of the diatom. The cultures were prepared by carefully isolating a few of the organisms from the original Plymouth sample and allowing them to grow in small flasks containing Miquel's culture fluid or sterilised sea water (see reference to Allen and Nelson's work). At the end of a month the bottom of the flask was covered with a thick brown growth of the diatom, but, as Allen and Nelson also observed, the cultures do not thrive well after more than a month so that at that stage the cultures were transferred to a number of large Kilner jars of 4-litre capacity containing the sterile culture fluid. The covered jars were allowed to stand in diffuse daylight in front of a window facing north, and the growth was accompanied by quite an appreciable evolution of oxygen on the brighter days. Frequent microscopical examination of the cultures demonstrated their freedom from contamination. By repeated sub-culturing in this manner large amounts of the organism were prepared without much trouble.

We were at first doubtful whether the administration of the fresh diatoms to rats in the usual manner of testing for the presence of vitamin *A* would be of any value but decided to test this method before going to the trouble of preparing by chemical means a special fraction. Somewhat to our surprise the organisms were digested during their passage through the alimentary canal of the rats as was indicated by the recovery of growth and by the presence of empty frustules in the faeces. The usual method of testing was followed [Drummond and Coward, 1920], the supplement being administered before the daily ration of basal diet was given. To prepare the organisms for administration the fluid was decanted off from a month-old culture and the thick flocculent growth of diatoms on the bottom filtered on to a thin layer of starch at the bottom of a Gooch crucible. After washing with a very small quantity of distilled water the almost dry mixture of starch and organisms was thoroughly mixed and an aliquot part given to the animals who invariably consumed it with readiness.

The organisms although possessing a deep brown colour whilst in culture assumed a dark green tint on drying. The total fat content (ether extract) was 5·25 % of the dry weight, and in addition to the characteristic pigment of the brown algae, fucoxanthin, there were also present chlorophyll and relatively large amounts of the lipochrome pigments carotene and xanthophyll.

Although it was impossible accurately to measure the dose of Nitzschia given daily an approximation was made on the basis of the average dry weight of a month-old culture which was 0·32 g.

The curves shown in Fig. 1 show that this diatom may serve as a source of vitamin *A*, the resumption of growth being especially marked at the higher dosage of approximately 0·08 g. (dry weight) per day.

The extraordinary potency of these diatoms as a source of the vitamin *A* may be judged from the fact that this ration of diatom represents approximately 4 mg. of fat; which recalls the observations of Zilva and Miura on the potency of cod liver oils [1921].

It is significant that this organism promoted growth very much more effectively than did the common seaweeds we previously tested. This difference may possibly be accounted for by taking into account the much greater relative surface of the diatoms.

The enormous stores, relatively speaking, of vitamin *A* which are found in the tissues of certain marine animals lead one to conclude that they are derived from some highly potent source of that factor such as is represented by the diatom we have been studying.

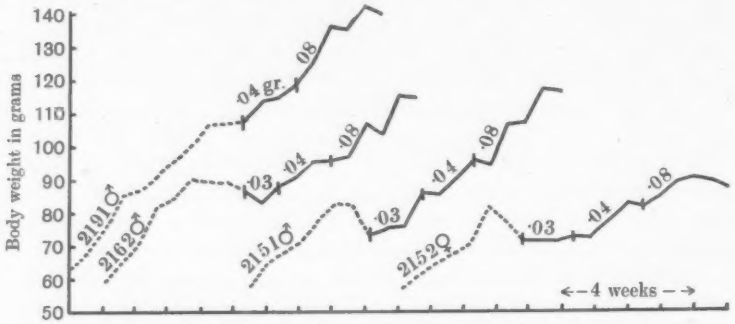

Fig. 1. Daily ration of Nitzschia (dry weight) shown in each period. Preparatory period shown by dotted line.

We essayed to prove the transference of the vitamin from the diatoms to the molluscs we were investigating but owing to the sad death of our colleague the experiments were carried no further than the demonstration that many species of molluscs are valuable sources of the *A* factor. The production of vitamin *A* in green land plants capable of carbon assimilation [Coward and Drummond, 1921] is therefore paralleled by a similar synthesis in marine plants containing photocatalytic pigments. Further, the fundamental dependence of terrestrial animal life on the fresh green leaf for its supplies of vitamin *A* would appear to be paralleled by a similar dependence of marine animals on the marine flora, particularly the microscopic plants.

Summary.

1. Pure cultures of a common marine diatom, *Nitzschia closterium*, grown in Miquel's solution or sterilised sea water synthesise large amounts of vitamin *A*.

2. A parallel is drawn between the dependence of land animals on fresh green leaves and that of marine animals on the synthetic activity of the marine flora for their supplies of vitamin *A*.

3. A number of molluscs were found to contain considerable amounts of vitamin *A*.

The greater part of the expense incurred in this research was defrayed out of a grant made by the Medical Research Council to whom our thanks are due. We also wish to express our appreciation of the assistance extended to us by the Ministry of Agriculture and Fisheries.

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XLI. THE PHOSPHOLIPIN OF THE BLOOD AND LIVER IN EXPERIMENTAL RICKETS IN DOGS.

By JOHN SMITH SHARPE.

From the Institute of Physiology, University of Glasgow.

(*Received May 1st, 1922.*)

In a paper in which he advances a theory of the metabolism in rickets, Prof. Noël Paton [1922] points out that recent work seems to indicate that the supply of phosphorus rather than of calcium may be the limiting factor in ossification and develops the idea that a disturbance in the metabolism of the phospholipins may be involved.

Since the phospholipins and especially lecithin play so important a part in the metabolism of phosphorus an attempt has been made to discover whether there is any modification in the amount in the blood and liver in rickets.

Unfortunately, the material available has not been of such a nature as to enable a conclusive answer to be given, for in each of the experiments in which the blood and liver were preserved for analysis, either all the pups remained free of rickets or all developed the condition. The results obtained are however sufficient to justify their being recorded and to allow a tentative conclusion to be drawn.

A considerable amount of work has been recorded upon the phospholipin of the blood but little of it has a bearing upon the present investigation. Bloor [1915] showed that in feeding with fat the lecithin content of the blood is raised and [1918] that this is due to an increase of phospholipin in the corpuscles.

Method.

The blood and the livers were taken at the time of death by chloroform and bleeding, from experiments 5, 6, 7 and 8 of the series described by Noël Paton and Watson [1921]. The details of experiment 12 have not yet been published, but all the pups remained free from rickets. Unfortunately the non-phospholipin phosphoric acid was determined only in lots 7 and 8.

Methods of Analysis.

Livers. The livers were preserved in formalin. A weighed amount was repeatedly extracted with alcohol-ether (10–1) by rubbing up in a mortar. The extract was dried in a steam-bath and in a vacuum desiccator and weighed and from this total fats and alcohol-ether soluble P_2O_5 were determined. The alcohol-ether-insoluble P_2O_5 was estimated in the residue. Each was ignited

Normal.

I. Pups of VII killed at 16 weeks old, those of VIII at 18 weeks old.

Experiments	VII. *B* 7		VIII. *B* 5		VIII. *B* 4		VII. *D* 4		VIII. *D* 2		VII. *D* 5	
Diet per diem	Separated milk (dried) 22·5 g. Bread 40 g.				Separated milk (dried) 22·5 g. Bread 30 g. Lard 4 g.						Full cream milk (dried) 22·5 g. Bread 30 g.	
Gain in weight %	620		280		240		600		300		366	
	Blood	Liver	Blood	Liver	Blood	Liver	Blood	Liver	Blood	Liver	Blood	Liver
Total fats %	2·94	2·19	1·60	1·2	1·25	2·45	2·12	3·4	2·04	2·45	2·34	2·9
Total P_2O_5 %	0·140	0·462	0·098	0·309	0·085	0·310	0·085	0·435	0·113	0·286	0·108	0·324
P_2O_5 in phospholipins %	0·049	0·098	0·035	0·096	0·027	0·161	0·026	0·167	0·038	0·109	0·042	0·115
P_2O_5 insoluble in alcohol %	0·091	0·364	0·063	0·213	0·058	0·149	0·059	0·268	0·075	0·177	0·066	0·209
P_2O_5 in phospholipins as % of total fats	1·7	4·5	2·1	8·0	2·1	6·5	1·2	4·9	1·9	4·4	1·8	3·9

II. Pups killed at 15 weeks old.

	1		2		XII 3		4		5	
Diet per diem	Full cream milk (dried) 22·5 g. increased to 34 g. Bread 30 g. increased to 50 g.									
Gain in weight %	200		120		83		200		50	
	Blood	Liver	Blood	Liver	Blood	Liver	Blood	Liver	Blood	Liver
Total fats %	1·86	5·5	1·80	5·8	1·32	6·7	2·15	—	1·90	—
P_2O_5 in phospholipins	0·052	0·209	0·059	0·186	0·048	0·320	0·052	—	0·042	—
P_2O_5 in phospholipins as % of total fats	2·8	3·8	3·2	3·2	3·6	4·7	2·4	—	2·2	—

Rickets.

Pups of V killed at 17 weeks old, those of VI at 13 weeks old.

Experiments	VI. *B* 5		V. *B* 1		V *B* 2		VI. *D* 2		VI. *B* 6		V. *D* 5		VI. *D* 3	
Diet per diem	Whole milk 250 cc. Oatmeal 45 g. after 5th week				Separated milk 250 cc. Oatmeal 45 g. after 5th week				Whole milk 250 cc. reduced to 150 cc. Oatmeal 45 g. after 5th week		Whole milk (dried) 30 g. Oatmeal 45 g. after 5th week		Separated milk 250 cc. reduced to 150 cc. Oatmeal 45 g. after 5th week	
Gain in weight %	156		66		22		116		161		135		65	
Condition	Slight rickets		Rickets (condition between V. *B* 2 and V. *D* 5)		Marked rickets		Rickets		Rickets		Slight rickets		Rickets	
	Blood	Liver	Blood	Liver	Blood	Liver	Blood	Liver	Blood	Liver	Blood	Liver	Blood	Liver
Total fats %	0·97	3·17	1·23	2·77	1·12	1·90	1·11	2·83	1·15	2·76	0·96	2·94	0·80	2·84
P_2O_5 in phospholipin %	0·022	0·076	0·033	0·072	0·027	0·068	0·024	0·090	0·026	0·084	0·024	0·100	0·019	0·084
P_2O_5 in phospholipin as % of total fats	2·2	2·4	2·7	2·6	2·4	3·5	2·1	3·2	2·3	3·0	2·5	3·3	2·3	3·3

with an excess of phosphorus-free calcium carbonate, the residue dissolved in hydrochloric acid with the addition of a little nitric acid and the phosphorus estimated by the molybdate method in the usual way.

Blood. The blood was drawn into a measured quantity of methylated spirit, well shaken and allowed to stand several days. This was filtered into a weighed flask and the residue extracted with alcohol-ether (10–1) many times, the washings being added to the filtrate. This was evaporated and the total fat and the P_2O_5 determined as in the liver.

Summary of Tables.

P_2O_5 in Phospholipin.

	Non-rachitic			Rachitic		
	Maximum	Average	Minimum	Maximum	Average	Minimum
Blood	0·059	0·043	0·026	0·033	0·025	0·019
Liver	0·320	0·162	0·096	0·100	0·082	0·068

Conclusions.

The tables show:

1. That the percentage of phospholipin in the blood and in the liver bears no relation to the gain in weight, to the amount of fat in the diet, to the total fat in blood and liver or to the age of the animal.

2. That in the rachitic pups the phospholipins of the blood and of the liver are lower than in the non-rachitic.

3. That the amount of phospholipins of the liver is smaller in proportion to that of the blood in rachitic than in non-rachitic animals.

I have to thank Prof. Noël Paton for the help and advice he gave me during the investigation. The expenses for the work were defrayed by a grant from the Medical Research Council to which body I tender my thanks.

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XLII. THE CONSTITUENTS OF THE FLOWERING TOPS OF *ARTEMISIA AFRA*, JACQ.

By JOHN AUGUSTUS GOODSON.

From the Wellcome Chemical Research Laboratories.

(*Received May 2nd, 1922.*)

The genus Artemisia comprises some 350 species, of which only about 30 have been chemically examined. In most cases the investigation has been confined to the essential oil, but a few of the more important species such as wormwood, *Artemisia Absinthium* Linn., used in the preparation of absinthe, and wormseed, *A. maritima* var. *Stechmanniana* Bess. which grows in Turkestan and is the sole source of the anthelmintic, santonin, have been more thoroughly examined. In consequence of the difficulty of obtaining santonin since 1914, other sources of this indispensable drug have been sought and in this connection attention has been given to other species of Artemisia, of which the author has examined four. *A. brevifolia* Wall. (which according to the Index Kewensis is a form of *A. maritima* Linn.) from India was found to contain santonin, as previously recorded by Greenish and Pearson [1921] and Simonsen [1921]; specimens of *A. afra* Jacq. from South Africa, *A. mexicana* Willd. from Mexico, and *A. monosperma* Delile (*A. deliliana* Bess.) from Egypt were found to contain no santonin. The closely related Egyptian plant *Santolina chamaecyparissa* was also found to be free from santonin.

Judging from these results and others previously recorded it would seem that the occurrence of santonin is restricted to species of Artemisia indigenous to East Europe and Asia, the only exception so far recorded being *A. gallica* Willd., which occurs in France, and according to Heckel and Schlagdenhauffen [1884] contains santonin. These authors, however, give no evidence for this statement and their observation has not been confirmed[1].

Of the four species examined by the author only one, *A. afra*, was available in quantity and this was fully investigated to see if it contained anything that could be regarded as a precursor or a derivative of santonin. The results given below show that this plant contains camphor, a wax-ester probably ceryl cerotate, triacontane, scopoletin and quebrachitol, none of which can be regarded as connected with santonin.

l-Camphor has been recorded in several species of Artemisia, *e.g.* in *A. Herba-alba* Ass. [Grimal, 1904], *A. cana* Pursh [Whittelsey, 1909] and *A. annua* Linn. [Yoshitomi, 1917]. In *A. afra* the camphor is dextrorotatory,

[1] Since this paper was submitted for publication a preliminary notice of a communication by Viehoever and Capen [1922] has appeared, in which it is stated that santonin has been isolated from *A. mexicana* and *A. neo-mexicana*. J. A. G.

but the rotation is much lower than that of normal *d*-camphor. Two specimens were found to have $[\alpha]_D^{20} + 9{\cdot}7°$ and $[\alpha]_D^{20} + 9{\cdot}3°$ instead of $[\alpha]_D^{20} + 42{\cdot}4°$. As it is usually considered that only two optically active isomerides can exist notwithstanding the fact that the camphor molecule contains two asymmetric carbon atoms, and as camphor is not easily racemised, it would appear that both *d*- and *l*-camphor are present in *A. afra*. It may be noted that inactive camphor has been found in *Chrysanthemum sinense* var. *japonicum* [Keimatsu, 1909].

Scopoletin and quebrachitol have not been recorded previously in the genus Artemisia or even in the natural order Compositae. The former has been found in *Atropa Belladonna* Linn. [Kunz, 1885], *Fabiana imbricata* Ruiz et Pav. [Kunz-Krause, 1899] and *Scopolia japonica* Maxim. [Eykman, 1884] of the natural order Solanaceae; *Ipomoea purga* Hayne (Convolvulaceae) [Power and Rogerson, 1910]; *Gelsemium sempervirens* Ait. (Loganiaceae) [Moore, 1910] and *Prunus serotina* Ehrh. (Rosaceae) [Power and Moore, 1909]. Quebrachitol has only been found in four other plants, viz. *Aspidosperma quebracho* Schlect (Apocyanaceae) [Tanret, 1889]; *Grevillea robusta* A. Cunn (Proteaceae) [Bourquelot and Fichtenholz, 1912]; *Heterodendrum oleaefolium* Desf. (Sapindaceae) [Petrie, 1918] and *Hevea brasiliensis* Muell. (Euphorbiaceae) [Pickles and Whitfield, 1911], all of which belong to different natural orders. The other components ceryl cerotate and triacontane are fairly widely distributed in plants.

Experimental.

For the material used in this investigation the author is indebted to Mr I. B. Pole Evans, Chief of the Division of Botany, Union of South Africa. It consisted of the flowering tops of the plant including stems, leaves and florets.

When extracted with Prollius's fluid it yielded a mere trace of alkaloid and furnished the following percentages of extract on exhaustion in a Soxhlet apparatus with solvents in the order named: petroleum (b.p. 35–60°), 7·0; ether, 9·5; chloroform, 2·1; ethyl acetate, 3·4; alcohol, 15·5. Special search was made for santonin with negative results. For the purpose of a more complete examination a quantity (11·1 kilograms) was extracted in succession with hot solvents, petroleum (b.p. 35–60°), ether and alcohol. The alcoholic extract was further fractionated by drying on a quantity of the flowering tops previously exhausted with petroleum and ether and re-extracting hot with chloroform, ethyl acetate and alcohol in succession. The petroleum extract on distillation in steam and extraction of the distillate with ether yielded 55·2 g. of essential oil; a further 28·6 g. was subsequently obtained from the ether extract, equivalent in all to 0·75 % by weight of the flowering tops.

The material left behind in the distillation flask on boiling with petroleum (b.p. 35 to 60°), deposited on cooling about 81 g. of crude wax-ester melting at 74–76°. The residue left on removal of the petroleum was boiled with ether

and this solution on cooling gave 17 g. of crude hydrocarbon, melting at 62 to 66°. The ethereal solution was then extracted with dilute hydrochloric acid, but yielded no alkaloid; it still contained some free and combined fatty acids, which were not investigated, and some unsaponifiable matter, which appeared to contain a sterol giving a red coloration with acetic anhydride and sulphuric acid.

Examination of the Essential Oil. The oil possessed the odour of camphor, had a specific gravity 0·9453 at 15°/15° and specific rotation $[\alpha]_D^{15} + 5{\cdot}8°$. On washing with solutions of sodium carbonate, sodium hydroxide and sodium bisulphite (saturated), it lost to each quantities of material too small to be investigated in detail. The acids extracted by sodium carbonate were fractionally precipitated as silver salts, the fractions containing 24·7, 34·9, 38·6, 39·9 and 41·3 % of silver respectively. Silver pelargonate, $C_8H_{17}COOAg$ requires Ag = 40·4. The saponification value of the oil before acetylation was 33·9 and after acetylation 73·9.

On distillation the following fractions per cent were obtained (1) below 180°/760 mm., 31·8; (2) below 100°/25 mm., 27·5; (3) at 100–120°/25 mm., 12·1; (4) at 120–140°/25 mm., 12·3; (5) at 140–180°/25 mm., 8·0; (6) and above 180°/25 mm., 7·7.

Isolation of Camphor. Fractions (2) and (3) on standing deposited camphor, a further quantity of which separated on redistilling the fractions and freezing the distillates with solid carbon dioxide, the total quantity obtained amounting to 13·5 % of the crude oil, but this does not represent the total amount of camphor in the oil as much still remained in the various fractions. The crude camphor was recrystallised from dilute alcohol until its melting point was constant at 178° (corr.) and showed no depression of melting point on admixture with natural *d*-camphor melting at 180° (corr.). The specific rotation was low, being only $[\alpha]_D^{20} + 9{\cdot}7°$ in 95 % alcohol ($\alpha_D^{20} = + 0{\cdot}98°$, $l = 1$ dcm. C = 10·148), that of natural *d*-camphor used for comparison being $[\alpha]_D^{20} + 42{\cdot}4°$ ($\alpha_D^{20} = + 4{\cdot}35°$, $l = 1$ dcm., C = 10·261). Found C = 78·9; H = 10·5. Calculated for camphor, $C_{10}H_{16}O$, C = 78·9; H = 10·6.

The oxime was prepared and after recrystallisation melted at 118–119° (corr.), the melting point remaining unchanged on admixture with camphor-oxime prepared from natural *d*-camphor. It was laevo-rotatory $[\alpha]_D - 3{\cdot}4°$ ($\alpha_D = - 0{\cdot}16°$, $l = 0{\cdot}5$ dcm., C = 9·296). The semicarbazone after recrystallisation from alcohol melted with decomposition at 241° (246° corr.) and when mixed with camphor-semicarbazone prepared from natural *d*-camphor, m.p. 246° (corr.) with decomposition, it melted at 242° (247° corr.). The melting point of the semicarbazone of *d*-camphor is usually given in the literature as 236–238°, but this is undoubtedly too low. (Found C = 63·4; H = 9·4. Calculated for camphor-semicarbazone, $C_{11}H_{19}N_3O$, C = 63·1; H = 9·2.)

Examination of wax-ester. The wax-ester was recrystallised many times from ethyl acetate or petroleum and then melted constantly at 79°, although

it appeared still to contain a small quantity of hydrocarbon. From the analyses of the ester and its hydrolytic products, it would appear to be ceryl cerotate or a closely related ester. (Found C = 82·5, 82·5; H = 13·5, 13·7. Calculated for ceryl cerotate, $C_{26}H_{53}COOC_{25}H_{51}$, C = 82·0; H = 13·8.)

The alcohol obtained on hydrolysis melted at 74°, and gave an acetyl derivative melting at 64–66°. Ceryl alcohol melts at 81°, and ceryl acetate at 64·5°. (Found C = 81·6, 81·5; H = 14·2, 13·9. Calculated for ceryl alcohol, $C_{26}H_{54}O$, C = 81·6; H = 14·2.)

(Found C = 79·0; H = 13·3. Calculated for ceryl acetate, $CH_3COOC_{26}H_{53}$, C = 79·2; H = 13·3.)

The fatty acid produced on hydrolysis melted at 76°. (Found C = 78·7, 78·8; H = 13·3, 13·5. Calculated for cerotic acid, $C_{26}H_{52}O_2$, C = 78·7; H = 13·2; m.p. 82°.)

Isolation of Triacontane. The crude hydrocarbon was distilled under reduced pressure and recrystallised several times from ethyl acetate and petroleum until it melted constantly at 66°. (Found C = 84·9; H = 14·9. Calculated for triacontane, $C_{30}H_{62}$, C = 85·2; H = 14·8; m.p. 65·5°.)

After removal of the essential oil, wax-ester and hydrocarbon from the ether extract, the latter was extracted with dilute hydrochloric acid, which removed no alkaloid or other basic material and then with sodium carbonate solution, followed by potassium hydroxide solution.

Isolation of Scopoletin. The sodium carbonate extract was acidified with hydrochloric acid, and extracted with ether. The ethereal solution on concentration deposited a crystalline substance, a further quantity of which was obtained by extracting the dilute hydrochloric acid extract of the original ether extract with ether. The substance was purified by recrystallisation from ethyl acetate, yielding 2 g. of pure material, which formed pale yellow needles and dissolved in sodium carbonate solution giving a solution possessing a striking blue fluorescence. It melted at 203° (208° corr.) the melting point being uninfluenced on admixture with scopoletin. (Found C = 62·4, 62·4; H = 4·2, 4·5. Calculated for scopoletin (4-hydroxy-5-methoxycoumarin), $C_{10}H_8O_4$, C = 62·5, H = 4·2.)

The chloroform extract yielded no crystalline substance and dilute hydrochloric acid removed from it only a trace of material giving alkaloid reactions.

Isolation of Quebrachitol (Methyl l-inositol). The residues of the ethyl acetate, and alcoholic extracts, after concentration, deposited a mixture of resinous matter and crystals, which was extracted with water, the solution was boiled with animal charcoal, filtered, and concentrated and finally alcohol was added when a quantity of crude methyl-*l*-inositol crystallised out corresponding to 0·43 % of the flowering tops used. After several recrystallisations it melted constantly at 194° (corr.), and had a specific rotation $[\alpha]_D$ − 81·6° (α_D = − 8·16°, l = 1 dcm., C = 10·0). Found C = 43·2, 43·4; H = 7·0, 7·3. Calculated for quebrachitol, $CH_3OC_6H_6(OH)_5$, C = 43·3; H = 7·3.

The acetyl derivative, prepared by the action of acetic anhydride in presence of pyridine, was recrystallised from ethyl acetate and obtained in rosettes of needles, melting at 94–95°. (Found C = 50·8; H = 6·1. Calculated for penta-acetylmethylinositol, $CH_3OC_6H_6(OOC.CH_3)_5$, C = 50·5; H = 5·9.)

There is no published record of previous chemical work on *Artemisia afra* but the author desires to state that Messrs H. W. B. Clewer, and R. R. Baxter have made preliminary examinations of the plant in these laboratories, and their records, which include the isolation of camphor and of the crystalline substance now shown to be quebrachitol, have been available to him.

In conclusion, the author desires to express his warmest thanks to Dr T. A. Henry for his advice and criticism throughout the course of the work.

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XLIII. A METHOD FOR THE ESTIMATION OF SMALL QUANTITIES OF CALCIUM.

By PATRICK PLAYFAIR LAIDLAW and WILFRED WALTER PAYNE.

From the Pathological Department, Guy's Hospital.

(*Received May 5th, 1922.*)

Our knowledge of calcium metabolism is limited, and probably hampered, by the lack of a good and accurate method of estimating small quantities of calcium. In quite a number of biological problems the estimation of amounts of calcium of the order of 0·1 mg. is frequently desired. The method about to be described is applicable to even smaller quantities than this; and is, we believe, remarkably accurate if used with reasonable care, and superior to those in use at the present time.

The modern methods are of two types (*a*) biological, (*b*) chemical. The biological methods depend on the fact that calcium is necessary for blood coagulation. Wright [1912] and later Vines [1921] have developed methods with this fundamental fact as basis. Inasmuch as the nature of the coagulation process is only imperfectly known these methods suffer from being built on an insecure foundation and are liable to break down for unknown reasons. They are, however, sensitive and within limits not clearly defined useful. If a method equally sensitive with a secure basis were available they would rarely be employed. The more recent chemical methods such as those of Howland and Kramer [1920], Kramer and Tisdall [1921] are difficult to use, and consisting as they do of microtitrations of oxalate by $N/100$ potassium permanganate are liable to error. Thus small amounts of organic matter such as carbon from imperfect incineration of a biological product, dust, or even the organic material in poor distilled water, may each and all cause significant error. The weak solution does not keep well, and the end point is not sharp. With elaborate precautions and in the hands of the select few they give good results; but we obtained very disappointing results on trying them out.

The principle of the method. The calcium is separated in the first place as oxalate and the oxalate is converted into calcium alizarinate under defined conditions. The crystalline calcium alizarinate is collected and washed on a Gooch filter and decomposed by oxalic acid. The alizarin is dissolved in alcohol made up to known volume with a relatively large volume of dilute ammonia, and the resultant solution of ammonium alizarinate is compared in a Dubosc colorimeter with a standard dilution of ammonium alizarinate. The primary

separation as oxalate is necessary since in spite of many trials no clean separation of magnesium and calcium alizarinates proved feasible. Since the calcium in the alizarinate is only one-sixth the weight of the alizarin, the initial weight of calcium to be estimated is multiplied by six; and since the colorimetric comparison is carried out against a 0·001 % solution of alizarin, the method is very sensitive. Given cleanliness, reagents free from calcium, pure alizarin, and avoiding undue hurry, 0·1 mg. calcium may be estimated, to 0·002 or 0·005 mg. without much trouble.

Precipitation of calcium oxalate. This is done along the usual lines and may be carried out on the ash of a biological product, or sometimes, as in the case of blood serum, directly. After careful incineration the ash is dissolved in 0·5 cc. of *N* HCl and transferred with about 2 cc. wash water to a centrifuge tube. One cc. of a saturated solution of ammonium oxalate is added, followed by 2 cc. of a saturated solution of sodium acetate, to which some ammonium oxalate has previously been added to exclude calcium contamination. The whole is mixed and allowed to stand for three hours, or if more convenient till next day. In our experience three hours' standing, especially in the case of direct estimations in blood serum, gives more uniform results than shorter periods, though most of the oxalate separates as a rule in a much shorter time. The oxalate precipitate is centrifugalised to the bottom of the tube (two minutes at 4000 per min. is sufficient) and the supernatant fluid sucked off as completely as possible without disturbing the oxalate precipitate at the bottom of the tube. Three cc. of a 0·1 % solution of ammonium oxalate are then added, the precipitate stirred up and the centrifugalisation repeated at once. The supernatant fluid is once more sucked off as completely as possible. In this way all the calcium is obtained as oxalate free from all but traces of magnesium, and with only a *little* excess of oxalate.

Conversion of oxalate into alizarinate. The oxalate is dissolved in 0·5 cc. of *N* HCl, and transferred to a clean test-tube. The centrifuge tube is washed out four times with water. The resultant dilute solution in the test-tube should measure 8 to 10 cc. A small volume is disadvantageous, as oxalate may separate at a later stage if the volume is kept too small. Excess of an alcoholic solution of alizarin is then added (1 cc. of a 1 in 1000 solution is sufficient for quantities of calcium of about 0·1 mg.). The test-tube is then warmed on a water bath to about 80° C., and five drops of strong ammonia solution added. The contents of the tube turn deep purple on mixing. It is best to add at this stage a minute amount of crystalline calcium alizarinate though it is not essential. The step ensures the full and ready separation of the calcium alizarinate in a crystalline form which facilitates filtration later. The tube is kept warm for about one hour then set aside to cool and stand till the following day. The whole of the calcium should then have separated in clumps of blue black microcrystalline needles at the bottom of the tube and the supernatant fluid should be pale in colour.

The calcium alizarinate is filtered off on a Gooch crucible with a fine asbestos

layer at a water pump, and the precipitate and filter washed with dilute ammonia. At this point care must be taken not to suck much air through the filter since acids, even the carbonic acid of the air, will decompose the calcium salt. Paper filters are not so good as asbestos. The ordinary papers contain significant amounts of salts which interfere; and extracted papers have their own difficulties.

The delivery from the Gooch crucible is now arranged to return fluid to the test-tube in which crystallisation of the alizarinate took place, and 1 cc. of a strong solution of oxalic acid in 50 % alcohol allowed to run all over the asbestos and precipitate. The calcium alizarinate decomposes at once and the blue black colour becomes orange. The alizarin set free is washed through with warm 95 % alcohol along with excess of oxalic acid into the test-tube. The asbestos should now be pink or very pale purple. The nature of this colour is unknown, but it does not appear to be due to any calcium compound and is usually greater when the oxalate is precipitated directly from blood serum, and less in the case of ash estimations. The alcoholic solution of alizarin is transferred to a 50 cc. standard flask, the test-tube washed out with dilute ammonia, the contents of the flask made just alkaline with ammonia, and the volume made up to the mark with water.

The standard solution for comparison is made up as follows. Two cubic centimetres of an accurate $M/1000$ solution of alizarin in alcohol are run into a 50 cc. standard flask, followed by 1 cc. of the oxalic acid solution in 50 % alcohol, and a volume of alcohol approximately equal to that used in extracting the alizarin from the Gooch crucible when dealing with the unknown. Water is added, the contents of the flask are made just alkaline with ammonia, and the volume made up to the mark. In this way a perfect colour match is readily obtained as the reactions of the test and standard solutions are nearly the same. A large excess of ammonia in one flask yields a purple tint as compared with a red one. A small difference in ammonia content is insignificant owing to the buffer effect of the oxalate.

The unknown and the standard solutions are then compared in a Dubosc colorimeter. 20 to 25 mm. depth of the standard solution yields a suitable tint for colour match.

The alizarin in the unknown solution is calculated from the depth of the column of fluid which gives a perfect match with the standard, and this figure divided by six gives the weight of the calcium.

The method is simple and though time must be spent over the whole process owing to the waits for complete precipitation at two stages, the actual time spent on manipulation is not great, and if a series of estimations is being done the time spent on any one is still smaller. It may be stated that there are many indications that if still smaller amounts than 0·1 mg. are in question, the use of small scale apparatus would enable these to be estimated with fair accuracy.

Experimental.

The composition of the calcium alizarinate as prepared from the oxalate, under the defined conditions of excess alizarin, in the presence of ammonia, and in weak alcoholic solutions, was determined by incineration of three separate specimens of the crystalline solid; conversion of the calcium carbonate so obtained to sulphate; and weighing after heating and cooling:

Alizarinate taken	Sulphate	Calcium	Theory
0·2424 g.	0·1023	0·0301	0·0307
0·4855 g.	0·2053	0·0604	0·0614
0·2535 g.	0·1111	0·0327	0·0321

0·2535 g. calcium alizarinate dried over sulphuric acid heated to

110° C. lost 0·0158 g.	$1H_2O = 0·0144$ g.
120° C. „ 0·0213 g.	
160° C. „ 0·0276 g.	$2H_2O = 0·0288$ g.
190° C. began to decompose.	

The weighing of the heated alizarinate was difficult since there was gain in weight during the weighings. The data show that under the conditions of experiment a salt of the composition $Ca_1Aliz_1 + 2H_2O$ separates. We also have evidence of the formation of another calcium salt of a different composition when there is not excess of alizarin present.

The method was tried out on an artificial salt mixture, made up to imitate the ash of blood serum but with the magnesium in excess, of the following composition:

NaCl	4·6340	
KH_2PO_4	0·0395	
$MgSO_4$ (dry)	0·7577	
$CaCO_3$	0·2410	
HCl	8 cc.	Water to 500 cc.

A trace of iron was present probably due to impurity in the magnesium sulphate. This was no disadvantage since this metal may be present in blood serum ash from haemoglobin.

The following table shows some of the results we obtained when estimating various amounts of the salt mixture:

Solution taken cc.	Ca found mg.	Calculated mg.	Difference mg.
0·9	0·171	0·1737	−0·0027
0·7	0·140	0·135	+0·005
0·6	0·117	0·115	+0·002
0·5	0·102	0·096	+0·006
0·4	0·0815	0·077	+0·0045
0·3	0·056	0·058	−0·002

Repeated estimations of quantities of about the amount normally present in blood serum gave the following figures:

Calcium taken mg.	Found mg.	Difference mg.
0·116	0·113	−0·003
0·116	0·118	+0·002
0·116	0·1196	+0·0036
0·096	0·098	+0·002
0·096	0·098	+0·002
0·096	0·096	0·000

Estimations on mixed samples of human blood serum are given below. It will be observed that the figures for the ash of the serum and the direct determinations are practically the same. Other figures show that the agreement is even closer if 24 hours be allowed for the separation of the oxalate in the case of the direct estimation. Other experiments on heated serum indicate that after heating some of the calcium becomes bound to the serum proteins and cannot be estimated directly:

	Ca in ash, mg.	Ca directly without ashing, mg.
Sample 1	0·098	0·096
	0·100	0·095
Sample 2	0·097	0·091
	0·094	0·093

SUMMARY.

A method for estimating small quantities of calcium is described. The method is colorimetric and depends on the insolubility of calcium alizarinate. It is accurate on 0·1 mg. to 0·002 mg.

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XLIV. PRODUCTION OF HYDROGEN PEROXIDE BY BACTERIA.

By JAMES WALTER McLEOD and JOHN GORDON.

From the Department of Pathology, University of Leeds.

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A study of phenomena of auto-inhibition in bacterial cultures was published by McLeod and Govenlock [1921] in 1921 showing that a series of investigations on this subject had led to the demonstration of such phenomena in connection with the growth of many bacteria. C. Eijkman's results [1904 and 1906, 1, 2], which have been more questioned than accepted, were fully confirmed.

It was shown further that the Pneumococcus, a bacterium, which did not appear to have been investigated previously in this respect, produced in the course of growth a substance inhibitory to its own growth and to that of most bacteria. This inhibitory effect was more powerful than that observed in connection with the growth of any other bacterium examined and the inhibitory substance was found to be thermolabile (85°). The labile character of the substance produced suggested an analogy with toxins and ferments and for this reason the name "bactericidins" was suggested for such substances. Subsequent work has shown however that the substance produced by the Pneumococcus, at all events, is simpler in character and is in all probability hydrogen peroxide: but the question whether other bacteria produce specific inhibitory substances analogous to ferments remains an open one.

A short summary of the work done to show that the Pneumococcus produces hydrogen peroxide in culture has already been published as an abstract of a communication to the Pathological Society [McLeod and Gordon, 1922].

It is the purpose of this paper to give in some detail the experimental evidence for this conclusion together with some more recent observations on the subject.

Conditions necessary for the production of pneumococcal inhibitory substance in fluid media.

The original observations establishing the existence of a thermolabile bactericidal product in pneumococcal cultures had been made in solid media-serum agar—and such were obviously unsuitable for attempting to concentrate the bactericidal substance.

In trying to obtain evidence of the production of these substances by the Pneumococcus in fluid media, the Staphylococcus was used as a test micro-organism because it grows freely and with great constancy in all ordinary bacteriological media and because it had proved to be particularly sensitive to the inhibitory body produced by the Pneumococcus.

The experiment consisted in preparing a series of dilutions in peptone water of a 10 % serum bouillon culture of Pneumococcus, which had been heated for 30 minutes at 60° to kill off the Pneumococcus, and inoculating these with Staphylococcus. The inhibitory potency of the pneumococcal culture was judged by the extent to which it had to be diluted in order to permit of growth of the Staphylococcus.

By using such methods it was soon determined that aeration of the culture was of importance; thus little or no inhibitory effect would be produced by growing a Pneumococcus in 10 cc. of 10 % serum broth in a narrow test-tube and the Pneumococcus was always alive at the end of 48 hours, whereas the same organism grown in 10 cc. of the same medium spread out over the base of an Erlenmeyer flask gave rise to a markedly inhibitory culture in which the Pneumococcus had usually died at the end of 48 hours. It was found further that inhibitory cultures could be obtained more constantly if a current of air was kept bubbling through the culture during incubation, by connecting the flask containing the medium with a water suction pump. Control observations in which air was passed through sterile media kept for similar periods of time in the incubator gave negative results—the medium did not develop any inhibitory quality.

All the earlier observations were made with a bouillon medium prepared from meat extract and Parke Davis peptone, 1 %, to which 10 % of horse serum was added. The horse serum had been heated for an hour at 56° and was several months old. It contained very little catalase and subsequent observations have shown that it is important for constant production of the inhibitory body that the medium should contain little or no catalase. Some of our observations also tended to show that the amount of inhibitory substance produced was influenced by the kind of peptone used but the point has not yet been investigated carefully enough to permit of a definite statement.

Methods of concentrating the inhibitory substance.

Under the impression that the substance under investigation was probably a labile protein similar to toxin an attempt was made to obtain concentration by precipitating out the proteins of the culture with absolute alcohol at 0°, and redissolving the precipitate in a small quantity of water. The solutions so obtained showed little or no power of inhibiting bacterial growth. Further experiment showed, however, that the bactericidal substance dissolved in alcohol and passed into the filtrate. By concentrating such filtrates *in vacuo* at 35°–45° fluids of considerably enhanced bactericidal potency were obtained.

The average culture before concentration had an antiseptic potency sufficient to prevent development of *Staphylococcus aureus* in peptone water containing from 1 part in 3 to 1 part in 8 of pneumococcal culture, and by concentrating this culture to one-sixth or one-eighth of its original volume in the manner outlined above a residue was obtained at least four times as active as the original culture, *i.e.* capable of completely inhibiting growth of Staphylococcus in peptone water if present in the proportion of 1 part to 20 of peptone water. That is to say the fluid obtained was about equal to 4 % carbolic acid as regards its antiseptic effect on *Staphylococcus aureus*: it differed from the latter however in the marked instability of the antiseptic substance contained.

By reprecipitating the first concentrate with an excess of alcohol and reducing to a considerably smaller bulk it was possible to obtain fluids of higher antiseptic potency.

Chemical evidence of the presence of peroxide in the inhibitory cultures and concentrates.

The antiseptic concentrates from pneumococcal cultures had not been long under investigation before the observation was made that the addition of such fluids to emulsions of blood was followed by evolution of gas. We are indebted to Mr H. D. Kay of the Physiology Department, Leeds University, for suggesting that the effervescence was probably due to the presence of peroxide. This appears to be the correct explanation. Both cultures and concentrates give a strong blue colour when mixed with starch iodide paste containing traces of $FeSO_4$ (Schönbein's reagent). Wolff [1912] has pointed out that under certain conditions nitrites may give this reaction and cause confusion in regard to presence of peroxides in plant juices; but the absence of nitrites from the cultures is proved by failure to obtain any colour reaction on adding diphenylamine. Cultures so concentrated also give quite definitely a yellow or orange colour with titanium sulphate solution. Lastly a compound blue in colour and soluble in ether but disappearing rapidly if not extracted with ether immediately was obtained on adding 10 % chromic acid to a concentrate.

The chief evidences from the chemical standpoint for considering this substance to be hydrogen peroxide rather than some organic peroxide are:

(1) Its sensitiveness to decomposition by the catalase of blood and serum or by special catalase preparations from the liver [Morgulis, 1921]; Novy and Freer [1902] state that the various organic peroxides with which they worked differed from H_2O_2 in being relatively insensitive to the action of catalase.

(2) The fact that it tends to decompose on heating, but only does so very rapidly when the temperature is raised to 85°, in which respect it closely resembles H_2O_2, which distils at 84° under reduced pressure when relatively pure and concentrated but is rapidly destroyed about the same temperature in dilute and impure solutions [Baur, 1908].

(3) The gas evolved on adding catalase to a concentrate was proved to be oxygen since it was not absorbed by KOH solution but was absorbed to the extent of 90–95 % by alkaline pyrogallol solution and was capable of rekindling a glowing splint placed in it. Further the oxygen evolved corresponded very nearly to the amount calculated as available when the concentrate was titrated with potassium iodide, sodium thiosulphate and starch with a view to determining its H_2O_2 content.

Bacteriological evidence of identity of inhibitory substance in pneumococcal cultures with H_2O_2.

Two lines of investigation have been pursued.

First of all a number of bacteria have been compared as regards their varying sensitiveness to the inhibitory effect of (*a*) small concentrations of H_2O_2 in agar plates (*b*) the substances diffusing from deep plate cultures of Pneumococcus to superimposed layers of agar [McLeod and Govenlock, 1921]. It has been found that if bacteria are graded according to the greatest concentration of H_2O_2 in an agar plate which is compatible with their growth the order of their resistance to the H_2O_2 is as follows: *B. subtilis*, *B. prodigiosus*, some Streptococci, *B. coli*, other Streptococci, *Staphylococcus aureus*, Anthrax, Cholera, Shiga, Typhoid.

Among the bacteria which were observed often enough on inhibitory pneumococcal plates to give a reliable average result the order of resistances was: Streptococcus, *B. coli*, Staphylococci and Shiga. Although complicating factors such as favouring effect of other products of pneumococcal growth on certain bacteria may be present, the results are remarkably alike.

The second line was to determine how far the antiseptic effect of the concentrate of a pneumococcal culture corresponded to that of a dilute solution of H_2O_2 of similar strength, *i.e.* one which would give a similar figure for available oxygen when that was determined by titration with potassium iodide and thiosulphate solution in presence of starch. Four experiments of this kind were performed and the results are given in Table I.

Table I.

Experiment	H_2O_2 of concentrated culture calculated in "volumes" by KI, $Na_2S_2O_3$ + starch titration	Antiseptic potencies expressed as highest dilution in peptone water which inhibited growth of *Staphylococcus aureus* completely			
		(*a*) Concentrated culture	(*b*) Solution of H_2O_2 in saline. Strength as calculated for concentrate	(*c*) Concentrated culture inactivated by heat : + H_2O_2 of same strength as in (*b*)	(*d*) Concentrated culture inactivated by heat
1	0·5	1–34	1–34	1–24	—
2	0·8	1–24	1–50	1–50	—
3	0·4	1–12	1–32	1–18	—
4	0·38	1–25	1–50	1–25	nil

It will be seen that the figures for antiseptic potency are nearly identical for concentrates and for heat inactivated concentrates which had received

addition of H_2O_2 sufficient to restore them to the same titration figure for available oxygen as that of the concentrates.

Further in the fourth experiment, where figures for concentrate and heat inactivated concentrate + calculated amount of H_2O_2 exactly correspond, a determination of volumes of gas liberated by catalase from 2 cc. of H_2O_2 of calculated strength was 0·7 cc. and that from 2 cc. of concentrate 0·67 cc.

That the antiseptic potencies of the concentrates should be usually less than those of solutions in 0·85 % saline of H_2O_2 of similar strengths is not surprising since in the strong solution of amino-acids etc. and suspended fatty bodies which constituted the concentrate the tendency to decomposition of the H_2O_2 is likely to be greater.

Changes in blood pigment effected by growth of Pneumococcus.

It has long been recognised that certain streptococci produce a green colour when grown on a blood agar plate, hence the name "viridans" [Schottmüller, 1903]. The Pneumococcus resembles this micro-organism in the appearance of its growth on blood agar plates. It has also been recognised for some time that pneumococci and certain streptococci produce methaemoglobin [Stadie, 1921].

Methaemoglobin is not however a green pigment and so far as we know no full investigation of the pigmentary changes associated with the development of the green colour has been made.

In recent years a medium called "chocolate agar" [Crowe, 1915, 1921; Neurin and Gurley, 1921], consisting of agar and heated blood mixed in varying proportions, has been much used. The blood here is no longer present in the form of haemoglobin or methaemoglobin and on such media the pigmentary changes produced by pneumococci etc. are much more striking than on unheated blood media. All gradations in colour between dark olive green and light yellow may be seen around pneumococcal colonies. If, however, small drops of dilute solutions of H_2O_2—$\frac{1}{4}$ to 1 "vol."—are repeatedly applied to the surface of such media a very similar range of colours is obtained. If the application of H_2O_2 is sufficiently powerful the medium is completely bleached. It would seem therefore that the varying colour changes which develop around pneumococcal colonies on such media are due to H_2O_2 formation in varying degrees of intensity. Also that the heated blood agar plate may reasonably be used to detect bacteria capable of forming H_2O_2.

Production of H_2O_2 by bacteria other than the Pneumococcus.

The following conclusions have been reached by using the "chocolate agar" plate as a primary indicator of H_2O_2 formation and then proceeding to confirm the fact that peroxide is produced by the bacteria which form green colonies, by growing them in bouillon or serum bouillon media in presence of abundant oxygen supply and testing their cultures with hydrogen peroxide reagents

such as Schönbein's or titanium sulphate solution. No bacteria have been met in the course of routine work or on going through a large series of stock cultures which produce a green colour on "chocolate agar," with the exception of various forms of cocci. Amongst the streptococci green-forming strains predominate but both amongst the haemolytic and the non-haemolytic forms strains are met which do not produce green on chocolate agar, and all such strains which have been examined have also failed to produce fluid cultures giving H_2O_2 reactions.

As a general rule, although with some distinct exceptions, the streptococci are differentiated from the pneumococci by producing a green colour later and with lesser intensity, similarly the presence of H_2O_2 is usually detected with the appropriate reagents at a later period in streptococcal than in pneumococcal culture, *i.e.* after 36 hours rather than after 18 hours.

In addition to the streptococci, bacteria from the urethra of sarcinal type and a coarse coccus unclassified have been found to produce a green coloration on "chocolate agar" and also to give H_2O_2 reactions in fluid media.

Amongst the many bacteria which do not produce green coloration on "chocolate agar" plates three were also tested for production of H_2O_2 in flasks of fluid media in which an air current was maintained. These were *B. coli*, Paratyphoid B. and Staphylococcus. All gave negative results and a number of different bacteria occurring from time to time as contaminations in such experiments have also given negative results.

Importance of catalase in connection with these phenomena.

It is an old finding in bacteriological work that most bacteria produce catalase [Gottstein, 1893].

If the production of H_2O_2 by bacteria as described above is a fact, certain observations may be reasonably expected to follow; amongst these are

(1) that bacteria producing H_2O_2 will not produce catalase;

(2) that such bacteria will grow much better in media containing catalase;

(3) that a medium containing some substance producing the catalase effect will be the best for maintaining stock cultures of such bacteria.

These observations have all been made; no evolution of oxygen over Pneumococcus colonies has ever been observed on pouring dilute solutions of H_2O_2 over cultures of these bacteria. The same holds for most streptococci. A more marked turbidity is developed in a serum bouillon culture of Pneumococcus than in a parallel culture where the serum bouillon has been heated to 65° for 30 minutes so as to destroy the catalase; that is if the cultures are incubated under conditions allowing of moderate access of oxygen. In fact it is very probable that the "vitamin" effects of fresh tissue fluids in promoting growth of Pneumococcus which Kligler [1919] describes are partly due to catalase.

Lastly it has been found that peptone bouillon containing 10 % of washed blood, a medium which retains to a considerable extent the power of de-

composing H_2O_2 even after heating for 20 minutes at 115°, is the most convenient for maintaining stock cultures of the Pneumococcus; no difficulty has been met in preserving various strains over long periods when subcultures have been made at 3–4 week intervals.

Possible importance of H_2O_2 formation by bacteria.

The most interesting possibility that arises in this connection is that H_2O_2-forming bacteria may under certain circumstances tend to kill themselves in the body by peroxide formation, just as they do in the test-tube. The most concrete case in which this possibility arises is the crisis in pneumonia. In the explanation of this there is admittedly a good deal of difficulty at the present moment, since the observed phenomena of phagocytosis, development of bactericidal quality in the serum, antitoxin formation etc. are inadequate. The chief difficulty in accepting such a theory of the crisis in pneumonia is that the pneumonic exudate is rich in catalase and it is most unlikely that any concentration of H_2O_2 similar to that which kills off the pneumococcus in culture can occur. There are two possible ways out of this difficulty. One is to suppose that something occurs to paralyse the catalase of the pneumonic exudate. Changes of reaction and enzyme reactions have been demonstrated in the pneumonic exudate which do not occur in the healthy tissues of the body [Lord, 1919, 1, 2; Lord and Nye, 1921, 1, 2]. Another is to suppose that a much smaller concentration of H_2O_2 may be effective in killing off the Pneumococcus in the lung than is required in cultural experiment on account of the oxidising ferments present in the exudate. Both however are subjects for extended experimental investigation.

Explanation of the formation of H_2O_2 by bacteria.

Wieland's [1912, 1, 2; 1913] theory of oxidation supposes that the essential phenomenon is the liberation of hydrogen and that the rôle of oxygen is that of an acceptor for the hydrogen liberated, while Wartenberg and Sieg [1920] show that, under certain conditions at all events, the first stage in the union of H and O is H_2O_2. Such a sequence of events would fit in well enough with the observed phenomena in pneumococcal cultures in which H_2O_2 is only formed as a bye-product where there is sufficient access of oxygen and little or no catalase.

The reason why H_2O_2 does not appear in the cultures of other bacteria may be either that they are catalase formers—the great majority of known bacteria; or that although not catalase formers they are too sensitive to the antiseptic action of low concentrations of H_2O_2 to grow sufficiently in the presence of oxygen to produce any recognisable traces of that substance—the anaerobes. When however certain strains of streptococci are considered which we have found to be incapable of producing catalase and relatively insensitive to H_2O_2, but which do not produce H_2O_2 in their cultures, it is obvious that other factors which await investigation must enter into the problem.

Conclusions and Summary.

1. The inhibitory substance developed in pneumococcal cultures to which there is abundant access of oxygen is H_2O_2.

2. This is proved both by chemical reactions and by the comparison of the antiseptic effects of the pneumococcal cultures and those of dilute solutions of H_2O_2 reckoned by titration etc. to contain a similar amount of available oxygen.

3. The early death of Pneumococcus in culture is usually due to accumulation of excess of H_2O_2.

4. The green or yellow colorations produced on heated blood media by certain bacteria are due to H_2O_2 formation.

5. In addition to pneumococci the only bacteria which have been shown to produce H_2O_2 are many streptococci, both haemolytic and non-haemolytic, and a few other coccal forms.

6. These findings can be utilised practically in putting bacteriological technique on a more definitely scientific basis in several respects.

7. The substance in fresh tissue fluids which specially promotes the growth of the Pneumococcus and which has been supposed to be of the nature of vitamin is most probably catalase.

In conclusion we have pleasure in expressing our indebtedness to Prof. M. J. Stewart in whose department this work was carried out and to Prof. H. S. Raper of Leeds University for valuable advice.

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XLV. NOTE ON URINARY TIDES AND EXCRETORY RHYTHM.

By JAMES ARGYLL CAMPBELL and THOMAS ARTHUR WEBSTER.

From the Department of Applied Physiology, National Institute for Medical Research.

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Introduction.

In previous papers [1921, 1922] we gave the results of observations on the composition of the day and night urine of the same subject under four different routines, each of five days duration. We showed that the differences between day and night urine were constant under a routine of complete rest in bed, a laboratory routine, a light muscular work routine and a severe muscular work routine. We found, in each case, that the total N, urea N, water and chloride were excreted in much greater amounts during the day than at night; that the amino-acids and uric acid were excreted in slightly greater amounts during the day than at night; that the acid, ammonia N and phosphate were excreted in much greater amounts during the night than during the day; and that creatinine and sulphate were more or less evenly distributed between day and night. In other words, there was an excretory rhythm which was not altered by any of the conditions of the four routines examined. In this rhythm there were definite tides during the day of water, chloride, total N and urea N, whilst at night there were phosphate, ammonia N and acid tides. It was considered that the greater excretion of acid, ammonia N and phosphate at night was due to delayed excretion of "certain fixed acids" formed in the cells during the day and that the phosphate tide at night was connected with the greater acidity at night.

To investigate further these various tides and this excretory rhythm we employed the same subject under further changes of routine. In the present paper we refer to differences between day and night urine, (1) during a routine of day starvation for 48 hours with food only at night, (2) during a routine of complete starvation for 24 hours and (3) during a reversed routine, for 96 hours, in which work was performed and food was taken during the night whilst the subject slept during the day.

Methods.

We have kept the methods constant throughout all routines.

Our subject took his usual diet but it was controlled to exclude articles which are known to influence greatly the composition of the urine and also to keep the average daily diet for each routine as similar in substance as possible. We employed his usual diet as we were undertaking a prolonged research in which the subject would at times be exposed to somewhat strenuous conditions. By making each routine cover several—and the same—consecutive days and by keeping the quality of the average daily diet constant, we thought that errors would be lessened. Moreover, preliminary observations under his normal daily diet showed that the differences between day and night urines were constant.

We collected the urine between 7 a.m. and 5 p.m. for the day and between 5 p.m. and 7 a.m. for the night. It was not considered that these periods were the best. They were chosen as being most convenient for our research. For a large part of this research the subject lived at the laboratory.

There is no accurate way to decide whether a waste product formed in a cell is excreted as soon as it is formed by the cell, or for how long the cell retains it. All periods for collecting urine have their advantages and disadvantages. The best results will probably be attained by a comparison of figures obtained during various periods of collection from each of many subjects under various conditions.

In estimating the acidity of the urine, we employed four methods—p_H, Folin's titration method, titration to p_H 7·4, and Leathes' acidity percentage method [1919]. As other observers have pointed out we found that the agreement between these methods as regards detail was not marked, but nevertheless the broad significance of the conclusion as regards acidity was similar with each method. Thus we were able to demonstrate the presence of the so-called alkaline tide in our subjects and to determine the difference in acidity between the day and night urines by any of these methods.

Starvation Routines.

Our subject starved all day taking food only between 5 p.m. and 10 p.m., the object of this experiment being to determine whether taking food at night would alter any of the tides, particularly the acidity and total N tides. For two days this routine was followed, and on the next day the subject starved for 24 hours. Table I shows the results for both these routines, namely, "day starvation" and "complete starvation." On comparing these results with our previous work [1921, 1922] it will be noticed that practically no change from our previous figures occurred as regards the differences between day and night urine. That is total N, urea N, water and chloride were still excreted in greater quantities during the day than at night, and acidity, phosphate and ammonia N were still higher at night than during the day.

Lusk [1917] records that the total N excretion during starvation was higher during the day than at night. From our results during starvation, it was obvious that the urine, under normal conditions, was not more acid during the night than during the day because taking of food during the day produced so-called alkaline tides. We have carried out many observations on our subject with regard to the so-called alkaline tide and found that any normal meal was followed by such a tide, in many cases the tide being well marked.

During the "day starvation" routine, our subject carried out sedentary laboratory work, whilst during the "complete starvation" routine he rested in bed reading most of the day.

We found about 20 mg. of creatinine N per hour in our subject, as seen in Table I. In our previous papers [1921, 1922] owing to an error we gave about 30 mg. per hour as the figure for this subject; all the figures in these previous papers for creatinine N should be reduced by one-third and the figures for undetermined N correspondingly increased. The general conclusions, however, are not disturbed by this correction, as the error was constant.

Reversed Routine.

In the "reversed routine" our subject worked and had most meals and fluid at night whilst he slept during the day from about 10.30 a.m. to 5.30 p.m. Meals were taken at 8 p.m., 2 a.m., 5 a.m. and 8 a.m. instead of the usual times 7.30 a.m., 1 p.m., 4 p.m. and 7.30 p.m. respectively. Four consecutive days of this "reversed routine" were spent by our subject C.P. and one of us (J.A.C.). Neither subject had become accustomed to the new routine by the fourth day; although both subjects had slept well during the day they felt very sleepy at night. Table I shows C.P.'s average results for the last 72 hours of this routine and Table II A gives J.A.C.'s average results. Average figures are given because the results for any one day were very similar to these. Subject C.P.'s results show that no change from the normal rhythm was obtained except that the phosphate for the day was much increased, the night phosphate remaining about the usual figure.

In the case of J.A.C. a similar increase of phosphate during the day was noted but also there were other changes; now the acidity and ammonia N were higher during the day when sleep was taken, so that the reversal of routine produced some reversal in composition in J.A.C.'s urine (see Table II A).

It is interesting to note that after four days of complete reversal of routine and meals (including fluid) the day and night partition of water and nitrogen and of practically all other excretions remained unchanged. This points towards a fixed physiological rhythm of the cells which cannot be altered easily. It is not likely that the kidney was responsible for this rhythm. All the body cells were probably responsible. The rhythm may be due to following closely a fixed daily routine—of work, meals and sleep—for a long period. Allowance for this phenomenon must, therefore, be made where necessary when carrying out experiments of this nature.

Table I. *Average hourly results. Subject C.P.*

	48 hours' day starvation (food between 5 p.m. and 10 p.m.)		24 hours' complete starvation		72 hours' reversed routine	
	Day (Sedentary work, no food)	Night (Food and sleep)	Day (Rest in bed)	Night (Sleep)	Day (Sleep)	Night (Work and food)
Amount cc.	56·0	25·0	88·0	17·9	71·3	34·5
Acidity %	46·4	78·6	33·0	58·3	42·4	70·4
Titratable acidity (Folin) cc. *N*/10	6·0	15·7	6·0	10·0	8·0	12·4
Total acidity, cc. *N*/10...	15·0	30·7	14·0	24·3	17·3	29·5
Total N g.	·379 (100)*	·307 (100)*	·392 (100)*	·195 (100)*	·373 (100)*	·337 (100)*
Urea N g.	·315 (83·00)	·248 (80·80)	·305 (77·80)	·142 (72·80)	·313 (84·00)	·250 (74·20)
Ammonia N (A) g. ...	·010 (2·64)	·019 (6·19)	·010 (2·55)	·018 (9·23)	·010 (2·68)	·018 (5·34)
Ammonia N (B) g. ...	·012	·021	·011	·020	·013	·024
Amino-acid N g. ...	·002 (0·52)	·002 (0·65)	·001 (0·25)	·002 (1·02)	·003 (0·80)	·006 (1·78)
Creatinine N g.	·020 (5·28)	·019 (6·19)	·019 (4·85)	·020 (10·25)	·021 (5·63)	·022 (6·53)
Uric acid N g.	·006 (1·58)	·004 (1·30)	·006 (1·53)	·003 (1·54)	·006 (1·61)	·007 (2·07)
Undetermined N g. ...	·026 (6·98)	·015 (4·87)	·050 (13·02)	·010 (5·16)	·020 (5·28)	·036 (10·08)
Chloride (NaCl) g. ...	·472	·213	·497	·134	·335	·261
Phosphate (P_2O_5) g. ...	·068	·082	·068	·078	·093	·081
Total S (SO_3) g. ...	·055 (100)	·068 (100)	·056 (100)	·041 (100)	·063 (100)	·072 (100)
Inorganic S (SO_3) g. ...	·039 (70·9)	·052 (76·5)	·044 (78·6)	·035 (85·4)	·054 (85·7)	·057 (79·2)
Ethereal S (SO_3) g. ...	·006 (10·9)	·009 (13·2)	·005 (8·9)	·001 (2·4)	·002 (3·2)	·006 (8·3)
Neutral S (SO_3) g. ...	·010 (18·2)	·008 (10·3)	·007 (12·5)	·005 (12·2)	·007 (11·1)	·009 (12·5)
Purine N g.	·0015	·0026	—	—	—	—
Calcium (CaO) g. ...	—	—	—	—	·011	·014

(A) Van Slyke's method. (B) Malfatti's method. Total acidity is ammonia (B) + titratable acidity.

* Figures in brackets are percentages.

Table II A. *Hourly averages. Subject J.A.C.*

	Reversed routine		Ordinary routine	
	Night Work and food	Day Sleep	Day Work and food	Night Sleep
Amount cc.	43·1	76·0	60·7	31·5
Acidity %	53·8	57·3	42·8	72·3
Titratable acidity, Folin cc. *N*/10	11·2	13·6	8·2	10·7
Total acidity cc. *N*/10	28·8	35·3	26·5	30·6
Total N g.	·408	·596	·533	·451
Ammonia N (B) g.	·025	·030	·026	·028
Phosphate (P_2O_5) g.	·074	·097	·055	·075

Table II B. *Hourly averages. Subject C.P.*

Experiment no.	Acidity %	Phosphate (P_2O_5) g.	Titratable acidity cc. *N*/10	Total acidity cc. *N*/10	Ammonia *N* (A) g.	Total N g.	Time	Sleep	Routine
1	20·6	·045	5·4	18·8	·014	·386	day	−	Rest in bed
2	70·2	·080	18·2	36·7	·022	·296	night	+	
3	44·8	·065	10·4	25·4	·015	·428	day	−	Ordinary
4	64·6	·080	16·8	33·0	·019	·332	night	+	
5	50·0	·066	11·6	28·1	·011	·447	day	−	Light muscular work
6	64·0	·075	15·4	31·6	·019	·357	night	+	
7	60·0	·070	14·0	29·0	·015	·446	day	−	Severe muscular work
8	70·0	·080	14·4	30·7	·019	·414	night	+	
9	46·4	·068	6·0	15·0	·010	·379	day	−	Day starvation
10	78·6	·082	15·7	30·7	·019	·307	night	+	
11	33·3	·068	6·0	14·0	·010	·392	day	−	Complete starvation
12	58·3	·078	10·0	24·3	·018	·195	night	+	
13	42·4	·093	8·0	17·3	·010	·373	day	+	Reversed
14	70·4	·081	12·4	29·5	·018	·337	night	−	

In both subjects the phosphate for the day and therefore for the whole 24 hours was distinctly increased. This increase was probably due to some special nervous metabolism connected either with sleep itself or with the desire to sleep. In Table II B, we have drawn up some results from all our experiments including those previously published [1921, 1922]. These show that a high phosphate excretion always accompanied sleep and that the higher of the day and night figures for phosphate excretion in each routine could be separated from every other factor in our experiments but sleep (see Table II B).

Sleep and Acidity of Urine.

In our previous papers we found that sleep was accompanied by a definite increase of ammonia, acidity and phosphate and a relative increase of sulphate. We suggested that there was a delayed excretion of "certain fixed acids" formed in the cells during the day, and that when formed in certain quantities, they were responsible for sleep and fatigue. In our present paper the higher figures for phosphate whether found by day or by night always accompanied sleep and sleep was independent of every other factor including acidity (see Table II B). Therefore, although these "certain fixed acids" might have been responsible for sleep and although higher acidity usually accompanied sleep it was not a necessary accompaniment of sleep (see experiment 13, Table II B). This is contrary to the finding of Leathes [1919], who considered that the CO_2 tension of alveolar air was increased at night above that for the day, as a result of depression of the respiratory centre during sleep. Collip [1920] also considered that an increase of C_H of the blood at night was due to sleep.

We see no reason to abandon the suggestion that sleep might be due to "certain fixed acids" since there was in the case of subject C.P. in the "reversed routine" an increased acidity of the urine previous to sleep although not accompanying it. He was obviously sleepy during the time of excretion of the larger amount of acid, but kept awake purposely. If the high acidity was due to "certain fixed acids" formed by the activity of the cells and capable of causing sleep, the cells would only recover after sleep itself and not after excretion of the "certain fixed acids." However, it was obvious that the subject had not become accustomed to the "reversed routine," so that our results really belonged to a transitional period and it is at present difficult to draw conclusions. We have carried out some observations on a subject who was somewhat more accustomed to work and food at night, with sleep during the day. He worked on a night shift every third week. The results we have obtained from him are in complete agreement with the results here described. Results from subjects accustomed to long periods of night work are required.

Phosphate Excretion.

We conclude from all our results regarding the higher figure for day and night phosphate excretion in each routine that it was intimately related to

metabolism either connected with sleep or occurring at the same time as sleep. We were able to separate the higher phosphate excretion in each routine from every factor, but sleep (see Table II B). Although, as a rule, the higher phosphate excretion accompanied the higher acid and the higher ammonia N excretion there was a marked exception to this rule (see experiment 13, Table II B).

Broadhurst and Leathes [1920] have suggested that the phosphate tide at night may be connected with some special muscular or nervous metabolism. Our previous results indicated that there was no connection with muscular metabolism and less clearly that there was none with nervous metabolism. Broadhurst and Leathes also found that the phosphate tide was not dependent upon food. Our results during the starvation routines confirm this finding. Fiske [1921], who considered that the phosphate tide was due in part to "retention of phosphorus," also showed that the phosphate curve was not affected by the taking of sodium bicarbonate so that the phosphate curve was not connected with excretion of acid. This may be the case, but there is much evidence from other observers that phosphate may have, as one of its functions, the removal of acid. Haldane [1921] found that acid was removed by phosphate if there was any phosphate available. In our subject C.P. a high acidity, as interpreted by a high titratable acidity and a high ammonia N excretion, was always accompanied by a high phosphate excretion.

We estimated the calcium in the urine and found that it was not connected with the increase of phosphate, the calcium being low when the phosphate was high and *vice versa*. When in the "reversed routine" the phosphate was absolutely increased, no such marked change was noted in the amount of calcium excreted.

According to text books [Hawk, 1919], some investigators hold that during extensive decomposition of nervous tissue the phosphate is increased. Mendel found that phosphate was increased after sleep produced by potassium bromide or chloral hydrate, so that it is possible that special metabolism connected with sleep was the main factor for the higher of the day and night phosphate excretions in our subject. Nervous metabolism may have been concerned.

It is interesting to note that in children we have found no such correlation; in them the phosphate followed closely the total N. In children, the condition is rendered more complex by the presence of growth metabolism together with the maintenance metabolism. Children did not show the same rhythm, probably because of different conditions with regard to sleep.

Summary.

1. Observations on day and night urine during a routine of day starvation, a routine of complete starvation and a reversed routine—that is with work and meals at night and with sleep during the day—are recorded.

2. Evidence was obtained that the urine was more acid at night, neither because the taking of food during the day produced alkaline tides, nor because the respiratory centre was depressed during sleep.

3. After four days of complete reversal of habit most of the differences between day and night urine still remained as before, so that there must be a fixed physiological rhythm connected with excretion by the body cells. Thus, although in the "reversed routine," most of the fluid was swallowed at night, the greater amount of urine was still excreted during the day which now included the sleep period. Also, although most of the food taken was eaten at night, the total N excretion still remained higher during the day than at night.

4. In the excretory rhythm referred to, the total N, urea N, water and chloride were excreted in greater amount during the day whilst the ammonia N, acidity and phosphate were higher during the night than during the day.

5. The higher figures for phosphate whether found by day or by night always accompanied sleep and could be separated from every other factor except sleep; and, with one exception, the higher phosphate excretion accompanied both the higher acidity and the higher ammonia N excretion in all routines examined. An absolute increase in phosphate occurred in the "reversed routine."

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XLVI. NOTE ON A NEW TANNASE FROM *ASPERGILLUS LUCHUENSIS*, INUI.

By MAXIMILIAN NIERENSTEIN.

From the Biochemical Laboratory, Chemical Department, University of Bristol.

(*Received May 2nd, 1922.*)

Paullinia tannin[1] is hydrolysed by emulsin, dextrose and β-gambier-catechincarboxylic acid being produced [Nierenstein, 1922]. It was therefore surprising to find that tannase, which had been prepared by growing *Aspergillus Luchuensis*, Inui in a solution of gallotannin and which is known to hydrolyse gallotannin into dextrose and gallic acid [Rhind and Smith, 1922], had no effect on paullinia tannin. This suggested the possibility that the hydrolysing properties of tannase depended on the medium in which it had been produced by the fungus and that a catechutannin, such as paullinia tannin, required a tannase, which had been formed in a solution of a catechu- and not gallotannin. This has actually been found to be the case, since *Aspergillus Luchuensis* yields in a medium in which gallotannin had been replaced by catechutannin from cube-gambier (*Ourouporia Gambier*, Baill), a new tannase, which hydrolyses paullinia tannin, but which has no action on gallotannin. We have therefore to distinguish between these two kinds of tannase and it is proposed to refer to them as *gallotannase* and *catechutannase* respectively. In this connection reference must be made to what may in time prove a dangerous practice, which has recently been introduced by Freudenberg and Vollbrecht [1921]. These chemists prepare tannase by growing an unknown species of *Aspergillus* in the aqueous extract of ground myrobalams, the fruit of *Terminalia chebula*, Retz. Such a medium must obviously affect the general properties of the tannase and ought to be avoided.

Freudenberg's method [1920], in which gallotannin is replaced by an aqueous solution of the residues of cube-gambier, from which the catechin is removed by extraction with ethyl acetate, is used for the preparation of catechutannase. The catechutannase thus obtained resembles gallotannase in

[1] This catechutannin is present in the seeds of *Paullinia Cupana*, H.B. and K., which is a synonym of *P. sorbilis*, Mart. These seeds are used for the preparation of the paste known as "pasta guaraná" and the tannin is consequently also referred to as guarana tannin [compare, for example, Perkin and Everest, 1918]. Harvey [1921] and also Procter [1922] have wrongly assigned the name *P. sorbilis* to "guara," which contains a gallotannin and not a catechutannin, as "guara" signifies *Cupania americana*, L. For this information I am indebted to the authorities of Kew Gardens, who also inform me that in Cuba the names "guaraná" and "guara" are apparently used for *Cupania macrophylla*, A. Rich. and *Guarea trichiliodes*, L. respectively.

appearance and produces when added to a solution of paullinia tannin (0·5 g. catechutannase are used for 2 g. paullinia tannin in 150 cc. of water to which 5 cc. of chloroform are added and the mixture kept in a dark incubator at 23° for ten days), a bulky precipitate, which when filtered off crystallises from water in small, pointed needles, which melt at 252–253°, carbon dioxide being evolved. These are in every respect identical with the inactive β-gambier-catechincarboxylic acid, previously obtained by the action of emulsin. (Found: C = 57·2; H = 4·6. Calculated: C = 57·5; H = 4·2 %.) For further identification a small quantity of the acid was methylated with diazomethane, when the corresponding pentamethoxy methyl ester was obtained. It crystallises from light petroleum in long needles, which melt at 74°. This melting point is not depressed on admixture with the same substance (m.p. 74°) previously obtained by the action of diazomethane on the acid.

The filtrate from the crude β-gambier-catechincarboxylic acid, freed from unchanged paullinia tannin with the aid of lead acetate and hydrogen sulphide, contains dextrose, which is identified as the dextrosazone (m.p. and mixed m.p. 200–203°).

In conclusion it is interesting to note that catechutannase produces the identical β-gambier-catechincarboxylic acid when added to the aqueous extract of fat-free cocoa-beans or to a solution of catechutannin from cube-gambier. This indicates a very close relationship between these tannins which has so far not been suspected.

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XLVII. A QUALITATIVE TANNIN TEST.

By ETHEL ATKINSON and EDITH OLIVE HAZLETON.

From the Biochemical Laboratory, Chemical Department, University of Bristol.

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Tanning consists in the fixation of the tannins by animal fibre. A reliable tannin test must therefore demonstrate this specific property and it is remarkable that no such test has so far been devised. The reactions which are generally used for the identification of the tannins, such as the colorations produced by iron salts or the precipitations which are given by potassium dichromate and gelatin [compare, for example, Onslow, 1920], obviously only indicate some incidental properties of the tannins. These reactions are also shared by other organic substances, thus phenols and hydroxybenzoic acids are known to give colorations with iron salts, whilst both gallic acid and gallotannin are precipitated by potassium dichromate [Drabble and Nierenstein, 1907]. Similarly gelatin is not only precipitated by tannins, but also by gum arabic [Pelletier, 1813], starch [Tollens, 1914], inulin [Tollens, 1914], methyl gallate [Nierenstein, 1905, 1912; Fischer, 1919; Freudenberg, 1920] and other substances. The want of a specific tannin test was therefore felt for many years in this laboratory, with the result that the following method has been devised by us at the suggestion of Dr Nierenstein.

A small piece of gold-beater's skin[1], about $\frac{1}{2}$ inch long and $\frac{3}{4}$ inch wide, is pinned on a flat surface of paraffin-wax, which is prepared by pouring melted paraffin-wax into a watch-glass. The skin is covered with a few cc. of water, in which it is left soaking for five minutes so as to make it better permeable to the tannins. The water is then poured off and the skin tanned by covering it with 1 cc. of a tannin solution, which is prepared by extracting for half-an-hour on a boiling-water bath 1 g. of the material to be tested with 50 cc. of water. After many experiments we have come to the conclusion that half-an hour's tanning suffices even for the weakest tannin solution, but that solutions of usual strength (about 1–2 %) tan in a few minutes. It is, however, advisable to tan not less than 15 minutes. The tanned skin is washed for two minutes at a constant drip of two drops per second and then stained for five

[1] Gold-beater's skin is the outside membrane of the large intestine of the ox. It has many advantages over other animal tissues. It is easily obtainable (the material used by us was supplied by the British Drug Houses) and it is very thin (1 yard × 6 inches weighs only about 3 g.). It consequently tans rapidly and requires only little tannin, which should make this test of use not only for chemical, but also for botanical work.

minutes with 1 cc. of a 1 % solution of ferric chloride. It is then again washed, dried and mounted for reference.

We find that only tanned gold-beater's skin is stained by ferric chloride. By this method we have been able to demonstrate the presence of tannins in the following plant products[1], all of which are known to contain tannins:

Aleppo galls, Chinese galls, Chinese plum galls, blue Basra galls, white Basra galls, Knopper galls, Sumac, Mangrove bark, Mimosa bark, Myrobalams, Valonia, Oak bark, Quebracho wood, Hemlock bark, Divi-Divi, Algarobilla, Canaigre, *Pistacia Lentiscus*, Golden Wattle, *Acacia Arabica*, Larch bark, Tea, Coffee, and Cocoa.

Gallotannin behaves exactly in the same manner, whereas gallic acid and pyrogallol, both of which are known not to possess tanning properties, are not fixed by gold-beater's skin, which is consequently not stained by ferric chloride. Identical results were also given by the following substances, all of which are known not to possess tanning properties:

Phenol, catechol, quinol, resorcinol, phloroglucinol, salicylic acid, protocatechuic acid and β-resorcylic acid.

The combination between the tannins and the gold-beater's skin is of a very permanent character. This is evident from the fact that the tanned and subsequently stained gold-beater's skin may be decolorised with dilute hydrochloric acid and again re-stained with ferric chloride. This process of decolorising and re-staining may be repeated several times without the slightest effect on the colour of the re-stained material.

In conclusion we would like to suggest that this test be referred to as the "gold-beater's skin test for tannins." We also wish to thank Prof. McCandlish of the University of Leeds and Mr M. C. Lamb of the Leathersellers' Technical College at London for the different tanning materials used in this investigation. Our thanks are also due to the Research Fund Committee of the Chemical Society for a grant allocated to Dr Nierenstein for his work on gallotannin, from which the expenses of the present investigation were defrayed.

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[1] We refrain from giving the Latin names of the plants we have used, since they are well-known tanning materials, which can be found in any book which deals with them [compare for example, Dekker, 1913].

XLVIII. THE ORIGIN OF THE VITAMIN *A* IN FISH OILS AND FISH LIVER OILS.

By JACK CECIL DRUMMOND and SYLVESTER SOLOMON ZILVA,

with the co-operation of

KATHARINE HOPE COWARD (*Beit Memorial Research Fellow*).

From the Biochemical Laboratories Institute of Physiology, University College, London, and Biochemical Department, Lister Institute, London.

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During the last two years the authors have been collaborating in an exhaustive investigation of the nutritive value of the edible oils and fats with particular reference to the dietary unit referred to as vitamin *A*. Of the time spent in this work a very large proportion has been devoted to the study of the oils of marine origin and in particular the fish liver oils, chiefly because it has been shown by Zilva and Miura [1921] that these oils represent by far the most valuable sources of the vitamin of all the groups into which the edible oils and fats are divided. Our study of the liver oils has been naturally divided into several phases each concerned with a distinct aspect of the subject and the present communication deals with the question of the origin of the relatively enormous stores of vitamin *A* which are found in the livers of many species of fish. The results set out in this paper supplement some recorded by Hjort who at our suggestion has studied a number of marine organisms in connection with this enquiry [1922].

It is now generally agreed that the higher land animals derive their supplies of vitamin *A* either directly or indirectly from green plants and the probability of a similar relationship existing in the sea has been suggested by Jameson, Drummond and Coward [1922]. The fundamental dependence of all marine animal life on the marine flora, particularly the microscopic flora, has been recognised for many years past, and the studies of Jameson, Drummond and Coward have shown that a typical marine diatom grown in pure culture can synthesise relatively large quantities of vitamin *A*, whilst Coward and Drummond [1921] showed earlier that higher forms of marine algae, whilst far less potent sources of this factor than diatoms, may contain a concentration approximately as high as that of typical land plants such as cabbage.

There appear to be no adequate grounds for doubting that the ultimate origin of the vitamin *A* in marine oils and liver oils is represented by the

marine plants possessing photocatalytic pigments, particularly those which are included in the term plankton. Our studies of fish eggs show us that the young fish normally begins its life with a considerable store of vitamin *A* derived from the yolk-sac. We have reasons for believing that there may be a transference of vitamin from the liver or tissues of the spawning fish to the eggs although as yet our experiments on this subject are incomplete.

Apart from our experimental work the existence of such a transference would appear probable from our knowledge concerning the transport of vitamin from tissues to the eggs or milk of certain terrestrial species.

Probably the vitamin-*A* content of the yolk-sacs is sufficient for the requirements of the developing larvae for some time, but it is well known that the period between the stage at which the contents of the yolk-sac are nearly absorbed and that at which the young fish are entirely dependent on external food is a very critical one.

The careful studies of Lebour [1918, 1919, 1, 2; 1920, 1921] and others have shown us that post larval fish feed very largely on copepods. Her work may for our purpose be summarised by quoting two paragraphs from one of her most valuable papers [1920].

"One finds that certain copepods and other entomostraca constitute by far the larger part of the food of nearly all the very young fish, and that usually each species of fish selects its own favourite food to which it keeps, indiscriminate feeding seldom or never taking place, and one can usually assign to each fish its own particular food." "Few fish are vegetarians, and it is unusual for any but the youngest fish to eat diatoms." "Very young herring or sprat and a few others often contain green remains which probably belong to some algae, and occasionally diatoms can be recognised in this but even before the yolk sac is absorbed the gut may contain larval molluscs and small crustaceans."

But whilst there is little or no direct feeding of fish on diatoms these and other microscopic organisms form the diet of the copepods and larval molluscs which play so important a rôle in the nutrition of young fish. The relationship between these two groups of organisms has been traced by a number of investigators with some care, but it is only necessary here to call attention to the very rapid multiplication of the plant life of the sea which takes place in the spring and which is intimately connected, as Moore has shown [1921], with the increase in light intensity during that period. Some short time after the rapid rise in diatoms and other plant organisms there is an associated rise in the numbers of microscopic animals, particularly copepods, and the occurrence of these two related phenomena has a very important bearing on the survival and development of the young fish hatched out at that season [Hjort, 1914].

By the kindness of Dr O. Borley of the Fisheries Laboratory, Lowestoft, we were enabled to study the vitamin-*A* content of a number of plankton samples collected during the spring of 1922.

The samples after being dredged, sifted through muslin and pressed, were preserved in an equal bulk of absolute alcohol and sent directly to us in London. On arrival they were evaporated to dryness *in vacuo* at 40° and ground well to mix the organisms thoroughly with the material extracted by the alcohol. The dry material was administered to rats in the usual manner for testing for vitamin *A*, and all four samples were found to be highly potent as sources of that factor when administered in daily supplements of 0·1 g. to the rats.

The four samples studied were representative of the staple food of a large majority of the small fish. The analysis of the samples for which we are indebted to Dr Borley is given below.

Table I. *Plankton samples.*

	1	2	3	4
Locality	52·2 N. × 3·8 E.	Sandetti Lightship 51·15 N. × 1·52 E.	Pas de Calais 50·58 N. × 1·30 E.	Pas de Calais 50·58 N. × 1·30 E.
Principal contents				
(1) Copepods	*Temora longicornis** Pseudocalanus elongatus Calanus finmarchicus Acartia longiremus	*Temora* *Pseudocalanus* Calanus Acartia Centrophages typicus	*Temora* Pseudocalanus Calanus	
(2) Mysids	Schistomysis spiritus Gastrosaccus spinifer		Macropsis Gastrosaccus	
(3) Amphipods	Paratylus swammerdami			
(4) Larvae	Decapod	Decapod	Decapod	Decapod
(5) Miscellaneous	Sagitta hexaptera Pleurobrachia	Oikiopleura Polynoid polychaetes	Sagitta	
(6) Post-larval fish	Herring			

* The species in italics were the most numerous in each sample.

Samples 1 and 2 were particularly interesting in that they consisted almost entirely of the very widespread genera of copepods Temora, Pseudocalanus and Calanus. These organisms directly or indirectly represent a very important stage in the food supply of the cod and thrive on diatoms such as *Nitzschia closterium* [Allen and Nelson, 1910], which has been shown to be a very rich source of vitamin *A* [cf. Jameson, Drummond and Coward, 1922].

Small fish. Having shown that the food of the majority of the small fish and certain other marine organisms is relatively rich in vitamin *A*, the next step was to study whether this factor is transferred to the tissues of the species which feed upon them. For this purpose we have taken one or two representative species from different groups without attempting to study the matter exhaustively, and have where possible selected those which most

frequently form the food of the cod and the related species which yield large quantities of marketable medicinal oils.

The food of the cod is not at all times the same. In Norway the early spring rise in diatoms leads to the vast multiplication of copepods, which form the food of enormous shoals of small fish which come in to the northern coast (Finmarken) to spawn. These shoals are chiefly composed of the caplin or capelan (*Mallotus vilosus*) and they are followed by great numbers of cod, coal-fish, haddock and other species which devour them in enormous quantities [Hjort, 1914]. Other species of young fish are frequently consumed by these larger species, but their diet also includes certain shell fish, larger crustacea, salps, and especially in the case of cod on the Newfoundland banks, squid.

The results of our few tests are given in Table II, but they only represent the testing of a few miscellaneous products we had on hand. No attempt has been made to examine these and similar products exhaustively.

Table II.

Species	Approximate daily dose to rats	Results	
Caplin, *Mallotus vilosus*	0·1 g.	Rapid growth	
Sprat, *Clupea sprattus*	1·0 g.	,,	,,
Young herring, *Clupea harengus*	0·4 g.	,,	,,
Mussel, *Mytilus edulis*	4·0 g.	Good	,,
Periwinkle, *Littorina littorea*	1·0 g.	,,	,,
Shrimp, *Crangon allmanni*	0·01 g.*	,,	,,

* Of unsaponifiable matter.

Of these species the periwinkle is interesting as being an example of a direct transference of the vitamin from the algae on which it feeds (Enteromorpha, etc.). The fat extracted from these animals after feeding is deeply coloured with chlorophyll and other pigments derived from these green weeds.

It would be an almost insuperable task to demonstrate an actual transference of vitamin *A* right through from a diatom such as Nitzschia to its final location in the liver of the cod, but it would appear that we are justified in assuming that such transference does indeed take place by relying on the fact that as yet synthesis of this dietary factor by an animal organism has not been demonstrated, whereas such synthesis is readily carried out by certain plants both marine and terrestrial. The dependence of marine animals on certain products of the synthetic powers of marine plants may indeed be as fundamental as that which exists between these two great groups of organisms on land, for many examples may be recalled such as that given by Fabre-Domergue and Bietrix [1900] who showed that a supply of microscopic plant life would save the lives of larval fish (soles) which were failing from malnutrition in spite of the fact that their yolk was not completely absorbed.

We have included in this paper a few of the unpublished results obtained with molluscs by the late Dr H. Lyster Jameson as well as the examination of certain material he had collected for other purposes since they have a certain bearing on the main subject.

SUMMARY.

1. The ultimate origin of the vitamin *A* found in the oils derived from fish, and particularly the fish liver oils, would appear to be chiefly the unicellular marine plants. Except very occasionally these organisms are not consumed directly by the fish.

2. The extraordinary rise in the number of marine plants which begins as soon as the intensity and duration of sunlight increase early in the year is followed by a rapid rise in the organisms, largely copepods, and larval decapods and molluscs, whose growth and development are dependent on their food supply which consists of minute plants. These minute animals, which form a large proportion of plankton, contain relatively large quantities of vitamin *A* presumably derived from the diatoms on which they have thriven.

3. The plankton forms the staple food of innumerable species of marine animals from small fish to some whales. This no doubt accounts for the presence of vitamin *A* in the tissues or fat depots of these animals.

4. The fish which yield the large bulk of the liver oils, cod, haddock, coal-fish, etc., feed on many species. At one season in northern Norway and Newfoundland they feed extensively on a small fish, the capelan or caplin, which has been found to be very rich in vitamin *A*, doubtless derived from its food.

5. The origin of the vitamin *A* in fish oils and fish liver oils has therefore been traced back to the synthetic powers of the marine algae which form the fundamental food supply of all marine animals.

It is a pleasure to acknowledge the valuable advice and assistance given to us by Dr Allen and Dr Lebour of the Marine Biological Laboratory, Plymouth, Dr Borley of the Fisheries Laboratory, Lowestoft, Professor Hjort and our late colleague Dr H. Lyster Jameson.

We also beg gratefully to acknowledge a financial grant from the Medical Research Council which defrayed the cost of this investigation.

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XLIX. THE RESPIRATORY EXCHANGE IN FRESH WATER FISH. III. GOLD FISH.

By JOHN ADDYMAN GARDNER, GEORGE KING and EDWIN BOOTH POWERS.

From the Physiological Laboratory, University of London, South Kensington.

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In part I of this series of papers [Gardner and Leatham, 1914, 1] a detailed account was given of apparatus for measuring the respiratory exchange in fish, and measurements were recorded showing the influence of temperature, size of the animal, etc. on the respiratory exchange in the case of brown trout.

In this paper we give an account of experiments by the same method on gold fish.

Method.

Preliminary experiments showed that gold fish live at a much lower plane of metabolism than trout, and it was found necessary to modify slightly the procedure adopted with the latter animals.

In the case of trout the duration of the experiments varied from four to six hours. Gold fish, however, both at low and at ordinary temperatures use much smaller volumes of oxygen per hour, and it was found necessary to continue the experiments over periods of 24 hours in order to obtain accurate measurements. At higher temperatures more oxygen was used and satisfactory results could be obtained in experiments of six hours' duration.

In most of the 24 hour experiments it was not found practicable to continue the pumping of the air through the water during the whole period, owing to irregularities during the night of the electric power used in working the pumps, though in one or two experiments the pumping was continuous. Usually the experiment was commenced about 11 a.m. and the pumping of the air through the water bottle was continued until as late as convenient in the evening. The pump was started again early in the morning and continued until the end of the experiment. Experience showed that the absorption of oxygen was so slow that, with the large bulk of water used, the tension of the dissolved oxygen was not reduced sufficiently during the time the pumps were out of action to affect the fish. Oxygen, equivalent to that used during the 24 hours, was gradually added to the air above the water in the experimental bottle during the period before the cessation of pumping.

To obtain accurate results by this method, it is essential to take the greatest care in measuring the temperature and pressure of the air above the water at the beginning and end of the experiment.

Some minor improvements were made in the thermostatic arrangements of the apparatus used for the trout, and the pressure gauges were fitted with spirit levels. Care was also taken to paint over all joints, and particularly the line of contact of the rubber bung and the neck of the bottle with a rapidly drying acid proof enamel paint. The gold fish used were about five to six inches long and averaged about 50 g. in weight. They were kept in a large tank outside in slowly running water. The tank had a gravel bottom and contained suitable water plants. The fish were fed in the usual way and remained perfectly healthy during the long period—more than a year—over which the experiments extended.

Experimental Results.

A detailed example of the method of calculation was given in the paper referred to above [1914, 1, pp. 379–380].

In the following protocols we give only details of the number and weight of fish used, the temperatures, and the *total* initial and final volumes of *free and combined carbon dioxide* of *oxygen* and of *nitrogen* in cc. reduced to 0° and 760. The volume of nitrogen should theoretically remain constant and the small positive and negative differences actually found afford a ready indication of the experimental errors.

Experiments at low temperature.

(1) Seven fish; weight 362 g.; individual variations 45–60 g. The fish prior to experiment had lived outside in cold weather. Initial temperature of water in experimental bottle 5·8°, final temperature 6·1°. Duration of experiment 22·43 hours.

CO_2	Initial	1352 cc.	Final 1459·4 cc.	Difference	+107·4 cc.
Oxygen	,,	5646·8	,, 5454·4	,,	−192·4
Nitrogen	,,	20356·4	,, 20349·3	,,	− 7·1

Nitrogen error −0·034 %.

(2) Seven fish; 348 g.; variations 42–59 g. I.T. 7·4°, F.T. 6·2°. These fish had been kept at from 3° to 6° for several days prior to the experiment. Duration 23·17 hours.

CO_2	Initial	1571·8 cc.	Final 1689·1 cc.	Difference	+117·3 cc.
Oxygen	,,	5162·2	,, 5069·1	,,	− 93·3
Nitrogen	,,	1868·8	,, 18705·4	,,	+ 18·6

Nitrogen error +0·099 %.

(3) Twelve fish; 601 g.; variations 38–65 g. These fish had had a long spell of mild winter weather prior to experiment. I.T. 6·15°, F.T. 1·4°. Duration 23·75 hours.

CO_2	Initial	1475 cc.	Final 1667 cc.	Difference	+192 cc
Oxygen	,,	5261·7	,, 5048·8	,,	−212·9
Nitrogen	,,	18991·9	,, 19036·7	,,	+ 44·8

Nitrogen error +0·23 %.

(4) Seven fish; 382 g.; variations 40–65 g. I.T. 6·2°, F.T. 6·3°. The fish had mild winter temperature prior to experiment. Duration 23·78 hours.

CO_2	Initial	2167 cc.	Final	2230·3 cc.	Difference +63·3 cc.
Oxygen	,,	4321·9	,,	4181·8	,, −140·1
Nitrogen	,,	15469·4	,,	15426·4	,, − 43·0

Nitrogen error −0·28 %.

(5) Seven fish; 368 g.; variations 40–60 g. These fish had been in water covered with a film of ice for the previous three days. I.T. 5·7°, F.T. 6·05°. Duration 23 hours.

CO_2	Initial	1648·6 cc.	Final	1781·5 cc.	Difference +132·9 cc.
Oxygen	,,	5216·4	,,	5091·2	,, −125·2
Nitrogen	,,	18894·4	,,	18884·1	,, − 10·3

Nitrogen error −0·054 %.

At medium temperatures.

(6) Seven fish; 355 g.; variations 35–60 g. I.T. 13·55°, F.T. 16·7°. Duration 24·25 hours.

CO_2	Initial	2066·9 cc.	Final	2459·2 cc.	Difference +392·3 cc.
Oxygen	,,	4427·2	,,	4020·7	,, −406·5
Nitrogen	,,	16044·3	,,	16017·6	,, − 26·7

Nitrogen error −0·17 %.

(7) Seven fish; 355 g.; variations 35–60 g. I.T. 13·55°, F.T. 15·9°. Duration 23·25 hours.

CO_2	Initial	2123·9 cc.	Final	2392·0 cc.	Difference +268·1 cc.
Oxygen	,,	4413·4	,,	3976·3	,, −437·1
Nitrogen	,,	15966·4	,,	15931·4	,, − 35·0

Nitrogen error −0·22 %.

At higher temperatures.

(8) Seven fish; 370 g.; variations 45–62 g. I.T. 21·4°, F.T. 20·6°. Duration 23·18 hours. In this experiment the valves of the pumping apparatus were out of order and the air could not be pumped through the water. At the end the water in the experimental bottle had a strong faecal smell.

CO_2	Initial	1518·4 cc.	Final	2256·5 cc.	Difference +738·1 cc.
Oxygen	,,	5098·7	,,	4521·2	,, −577·5
Nitrogen	,,	18551·2	,,	18519·1	,, − 32·1

Nitrogen error 0·17 %.

(9) Seven fish; weight 361 g.; variations 45–63 g. I.T. 20·7°, F.T. 20·4°. Duration seven hours. At the end no objectionable smell was noted.

CO_2	Initial	1649·1 cc.	Final	1855·2 cc.	Difference +206·1 cc.
Oxygen	,,	4640·8	,,	4354·0	,, −286·8
Nitrogen	,,	16805·0	,,	16819·9	,, + 14·9

Nitrogen error +0·088 %.

The results of these experiments are given in Table I.

Table I.

No. of exp.	Average weight of fish used in g.	Volume of oxygen consumed per fish per hour in cc. at 0° and 760	Volume of CO_2 produced per fish per hour in cc. at 0° and 760	Volume of oxygen consumed per kilo of fish per hour in cc. at 0° and 760	Volume of CO_2 produced per kilo of fish per hour in cc. at 0° and 760	Respiratory quotient
At low temperature:						
(1)	51·7	1·225	0·684	23·70	13·23	0·56
(2)	49·7	0·574	0·723	11·56	14·55	1·26
(3)	50·0	0·747	0·674	14·91	13·45	0·98
(4)	54·5	0·842	0·380	15·42	6·97	0·45
(5)	52·5	0·778	0·825	14·79	15·70	1·06
	Mean	0·833	0·657	16·07	12·78	
At medium temperature:						
(6)	50·7	2·395	2·309	47·22	45·54	0·97
(7)	50·7	2·686	1·647	52·96	32·48	0·61
	Mean	2·541	1·978	50·09	39·01	
At higher temperature:						
(8)	52·8	3·560	4·550	67·35	86·08	1·28
(9)	51·6	5·853	4·207	113·50	81·55	0·72
	Mean	4·706	4·379	90·43	83·81	

Influence of Temperature.

It will be seen from Table I that the volume of oxygen consumed per fish, or per kilo of fish, increases with the temperature and very roughly in proportion to the temperature.

We should scarcely expect to find any exact proportion in experiments on the living animal, more especially as, owing to the long period over which the work extended, different sets of fish were used, and some latitude must be allowed for individual idiosyncrasy. Further in most of the experiments it was necessary to keep the animals in the experimental bottle for about 24 hours and during some hours the pumping of air was discontinued, so that during some part of the experiment the oxygen tension in the water would be below saturation. Experiments 8 and 9 at higher temperatures need some comment, as they show marked difference in the oxygen absorption, though most of the fish used were the same in each.

In experiment 8, which was of 24 hours' duration, the valves of the apparatus went wrong at the beginning, so that it was impossible to pump the air of the bottle through the water. Consequently the oxygen tension in the water progressively decreased, and at the end the partial pressure of the oxygen in the water was only 0·583 % of an atmosphere. At the end it was also noticed that the water had an objectionable faecal smell, a condition not found in any of the other experiments. It seemed probable therefore that the oxygen consumption figure was below normal. The experiment was therefore repeated (No. 9) and the duration was reduced to seven hours. Pumping was continuous. The oxygen figure is markedly higher than in No 8, and the respiratory quotient approximately normal for an unfed animal.

In the experiments at low and at normal temperatures, owing to the relatively slow oxygen absorption of the fish, it did not seem likely that the

small reduction of the oxygen tension in the water during the non-pumping stage of the experiment would have much effect.

To test this, however, we made the following experiment at a moderate temperature keeping the oxygen tension below normal during the whole time.

(10) Seven fish; weight 329 g.; variations 22–65 g. I.T. of water 16·8°, F.T. 16°. The water had been previously partially denuded of oxygen and the gas over the water was air and nitrogen. Duration six hours. The partial pressure of the dissolved oxygen was found to be at the beginning 6·17 % and at the end 2·69 % of an atmosphere.

CO_2	Initial	436·0 cc.	Final	510·1 cc.	Difference	+ 74·1 cc.
Oxygen	,,	2301·9	,,	2168·2	,,	− 133·7
Nitrogen	,,	23818·5	,,	23847·6	,,	+ 29·1

Nitrogen error +0·12 %.

Oxygen absorbed per fish per hour 3·114 cc. or per kilo of fish per hour 66·26 cc. Carbon dioxide evolved per fish per hour 1·726 cc., or per kilo per hour 36·72 cc. The respiratory quotient is 0·55.

At the temperature used the oxygen absorption should be a little higher than the mean of experiments 6 and 7. This is evidently the case, and we may conclude that the non-pumping interval in the experiments at low and at medium temperatures did not appreciably affect the result.

Comparison of the oxygen requirements of trout and gold fish.

In Table II the oxygen requirements of trout and gold fish, at various temperatures, are compared. The gold fish were 5 to 6 inches in length, and the sets of trout 4 inches and 8 inches respectively, but comparisons in terms of length are somewhat imperfect, owing to the difference in shape of the two kinds of fish. We were unable to obtain gold fish of the same average weight as the larger trout, in sufficient number. A glance at the table will

Table II. *Comparison of oxygen consumption of gold fish and trout.*

	4 inch trout, average weight 23 g.		8 inch trout, average weight 102 g.		5 to 6 inch gold fish, average weight 51 g.	
	Per fish per hour	Per kilo of fish	Per fish	Per kilo	Per fish	Per kilo
4°–7°	2·05	89·04	10·48	102·53	0·83	16·07
13°–16°	2·82	117·40	17·84	192·36	2·54	50·09
20°–22°	4·63	213·80	19·57	204·24	4·71	90·42
25°	5·64	258·87	—	—	—	—

show that the gold fish live at a much lower plane of metabolism than trout, and require much less oxygen. They also appear to react to temperature over a wider range than trout. At temperatures round 16° gold fish use about three times as much oxygen as at 4° to 7°, whereas the 8 inch trout use about double. A further rise in temperature from about 16° to between 20° and 22° again doubled the oxygen used by the gold fish, but caused only a small increase in the case of trout. It would appear at first sight that in the case of the large trout the organism ceases to react to rise of temperature about

the limit of 16° to 17°, but that in the case of gold fish this limit is higher, for the ratio of oxygen consumption of gold fish to trout at 4° to 7° is about 1:6, at about 16° 1:4, and at 20° to 23° nearly 1:2. It is probable, however, that this difference in reactivity to temperature is more apparent than real, for it has been shown [Gardner and Leatham, 1914, 2] that trout can live and keep well for long periods at a lower level of oxygen partial pressure than that corresponding to full saturation, and it is well known that gold fish flourish at very much lower levels of saturation; in addition the large trout were somewhat restricted in their movements in the experimental bottle compared with the smaller animals, and were consequently less favourably situated for assisting the pumping of water through the gills by swimming movements. If we make the assumption that a fish absorbs the whole of the oxygen from the water passing through its gills, then calculating on the basis of Winkler's figures [1905] for the oxygen content of water saturated with air at various temperatures, a 4 inch trout at 7° to 8° would need to pump about 4 cc. per minute through its gills, while at 21° he would need about 12·4 cc. per minute and at 25°, 16 cc. per minute. On the same basis an 8 inch trout would need to pump at 6°, 20 cc. per minute, and 16°, 44 to 45 cc. The above assumption is perhaps scarcely warrantable, so that these figures must be regarded as minimum values. It is therefore perhaps not surprising that the large fish should apparently cease to react to increases of temperature at a lower temperature than the small fish.

Another factor which appears to bear on the difference between trout and gold fish in this respect is that gold fish are able to bear with impunity higher temperatures than trout. As was shown in the paper referred to above [1914, 1] temperatures round about 25° appear to be dangerous or fatal to trout. Measurements of respiratory exchange were successfully carried out on 4 inch trout at 25°, but on attempting to determine the oxygen absorption for 8 inch trout at this temperature, of the three fish used in the experiment, one died in about two minutes, though the water was fully oxygenated, and the other two turned over on their backs in about ten minutes, and would no doubt have died, but on adding cold water they recovered completely and rapidly, and appeared to be quite well the next day. This result was not due to any sudden change of temperature, as the fish were carefully and gradually warmed up beforehand during the course of one hour. In another experiment four 8 inch fish were accidentally plunged into well-oxygenated water at 33°. The fish gave a few violent leaps and collapsed as one might perhaps imagine a warm-blooded animal doing on falling into boiling water, and were all quite dead in under one minute. The water was afterwards found to be quite frothy, as though it contained some saponaceous material. Some experiments were made with gold fish to ascertain the maximum temperature compatible with life. A strong healthy gold fish (about 50 g.) was placed in a large earthenware basin of water at 20°. After 15 minutes warm water was carefully added, with good stirring, until the temperature rose to 25°. At intervals of 10–15

minutes more hot water was gradually added, and the temperature very gradually increased. At 30° the fish was very active, swimming about more rapidly and continuously than at lower temperatures, but beyond this showed no apparent sign of distress, though the breathing, as one would expect, was rather rapid. At 35° the fish apparently died without any struggle, remaining on his side at the surface, the mouth being closed. No sign of life being observed after ten minutes at this temperature, the water was then gradually cooled down to 17°. After about one hour the fish was observed to be breathing feebly. It then began to recover more rapidly; it was put into the store tank outside, and the next day appeared to have completely recovered.

It would seem from these experiments that trout can live in water up to a temperature of from 20–25°, but gold fish can exist without apparent harm up to at least 30° or even higher. This result is, we believe, in accordance with the geographical distribution of these fish. As far as we are aware trout are never found in tropical rivers, except such as have a snow mountain source.

Respiratory Quotient.

We are not yet able to explain the variations noted in the respiratory quotient, particularly the very low values observed with trout at low temperatures, but a careful examination of our experimental results has convinced us that the variations cannot be attributed to experimental error.

Experiments are in progress which we hope will throw light on this point.

We take this opportunity of expressing our thanks to the Government Grant Committee of the Royal Society for aid in carrying out this work.

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L. NOTES ON SOME PROPERTIES OF DIALYSED GELATIN.

By DOROTHY JORDAN LLOYD.

From the Biochemical Laboratory, Cambridge, and the Laboratory of the British Leather Manufacturer's Association.

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I. *Material.*

The gelatin on which the following observations was made was purified by dialysis at the iso-electric point ($p_H = 4{\cdot}6$) and subsequent precipitation in strong alcohol. Details have been published in a previous paper [Jordan Lloyd, 1920]. The dry gelatin contained 0·06 to 0·00 % of ash. It was obtained as a snow white, fibrous and brittle solid, with a low solubility. In one specimen the maximum solubility was 1·5 % in boiling distilled water. In others 2–2·5 % could be dissolved under the same conditions, but in no case was it possible to dissolve more than 2·5 % of the pure fibrous gelatin in pure boiling water. This agrees with the observations of Dheré [1910] and Dheré and Gorgolewski [1913] that prolonged dialysis is accompanied by diminished ash content and decreased solubility. The hot water solutions were clear and colourless. On cooling to 15°, if the concentration were above 0·9 % the sol set to a turbid white gel in 24 hours, below 0·9 % but above 0·6 %, gelation took from 2–5 days. At a concentration of 0·6 % or less, the clear hot sols cooled to form turbid white sols at 15°, the turbidity decreasing with decreasing gelatin content. The gelatin in these sols is not precipitated by ferric, mercuric or lead salts, nor by ferrocyanides, dichromates or tannic acid. Gels formed under these conditions are not stable, the gelatin separating from the water [see Jordan Lloyd, 1920; Smith, 1921]. The solubility of dialysed gelatin is, as would be anticipated, immediately increased by the addition of acid or alkali. Gels formed in the presence of free acid or base are glassy clear, colourless and stable.

II. *The influence of hydrochloric acid, sodium hydroxide and sodium chloride on the gelling power.*

"Gelling power" is a difficult phrase to define precisely, and the property which it purports to describe is equally difficult to measure accurately. The following notes do not claim to set out any satisfactory measurement or definition of gelling power, the differences described between the experimental fluids being purely comparative, and observed under conditions kept as

uniform as possible. The solutions of gelatin used for this comparative work were made by dissolving dialysed gelatin in hot water, raising to boiling point, and then allowing to stand for 48 hours at 15°. The minimum concentration of gelatin required to produce a gel under these conditions was assumed to give an inverse measure of the gelling power. A fluid was taken as gelled when it remained in its place in an inverted test-tube. The influence of hydrochloric acid and sodium hydroxide was examined in the absence of sodium chloride and in the presence of 0·1 *N*, 0·4 *N* and *N* sodium chloride. The reactions of the 1 % gelatin solutions were measured electrometrically and also by adding a few drops of indicator at the time of mixing, and comparing the colours with suitable standards at the end of the experiment. It is well known that the addition of sodium chloride to a solution containing hydrochloric acid increases the concentration of hydrogen ion but Harned [1915] has shown that the increase is not great. In the present case it will be well within the experimental error. The measurements of reaction are accurate to $\pm$ ·2 p_H. The results of the experiment are summarised in Tables II, III, IV and V, and a diagram, in which the minimum concentration that will cause gelling is plotted against reaction, is given in Fig. 1. Areas above the curves are sols, those below are gels.

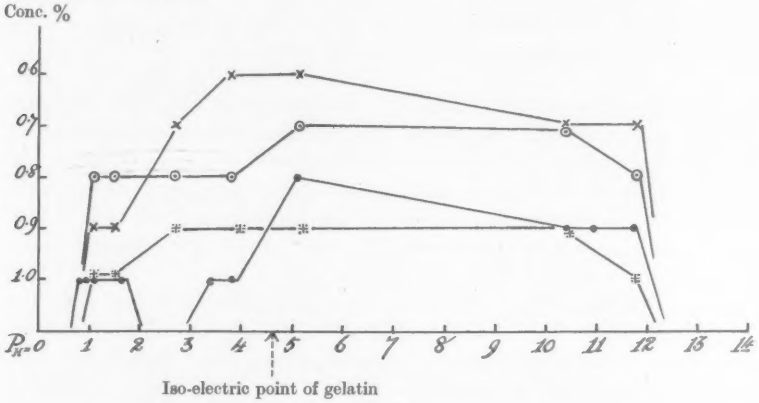

Fig. 1.

Abscissae = p_H.
Ordinates = Minimum concentration per cent. at which gel forms. 15° and 48 hours.
●——● = 0·0 *N* NaCl ⊙——⊙ = 0·4 *N* NaCl
×——× = 0·1 *N* NaCl ⁂——⁂ = 1·0 *N* NaCl

Pure gelatin under the given conditions (48 hours' standing at 15°) requires a minimum concentration of 0·8 % to form a gel, and will only form gels at 1 % concentration from p_H = 12–3, and again rather surprisingly between p_H 2 and 0·7. The disappearance of the power of gelation in acid solutions and its temporary reappearance in stronger acids is a phenomenon which has not previously been described. The conditions necessary to observe it are rather restricted, temperature being an important factor as is shown in Table I.

Table I.

p_H	Concentration of HCl or NaOH	1 % gelatin after 48 hours		
		0° C.	15° C.	20° C.
0·7	·25 *N* HCl	—	Clear fluid	—
0·8	·20	Transparent fluid	„ very soft gel	Clear fluid
0·9	·15	„ „	„ soft gel	„ „
1·1	·10	„ „	„ gel	„ „
1·5	·05	„ soft gel	„ „	„ „
1·6	·04	—	„ soft gel	—
1·7	·03	—	„ „	—
2·0	·02	—	„ fluid	—
2·8	·01	Transparent fluid	„ „	Clear fluid
3·8	·005	„ soft gel	„ soft gel	„ „
	·002	Gel with ice crystals	—	„ gel
	·0025	—	Clear gel	—
	·0015	Very faintly turbid gel	—	Clear gel
	·0010	Faintly turbid gel	—	Faintly turbid gel
	·0005	Turbid gel; hysteresis	—	Turbid gel
5·1	Distilled water	Opaque white gel	Opaque white gel	„ „
	·0005 *N* NaOH	Faintly turbid gel	—	Very faintly turbid gel
	·0010	Very faintly turbid gel	—	Clear gel
	·0015	„ „	—	„ „
	·002	„ „	—	„ „
10·4	·005	Clear gel	Clear gel	„ fluid
11·7	·01	„ viscous fluid	„ „	„ „
12·15	·02	—	„ very soft gel	—
12·4	·03	—	„ viscous fluid	—
12·5	·04	—	„ fluid	—
12·6	·05	Clear fluid	„ „	—
12·9	·10	„ „	„ „	Clear fluid
13·1	·20	„ „	„ „	„ „

At concentrations of 0·1 *N*, and 0·4 *N*, sodium chloride lowers the minimum concentration of gelatin required to produce gelation, except at reactions more alkaline than $p_H = 12·2$ where in no instance was a gel obtained. At normal concentration sodium chloride may either increase or decrease the gelling power, according to the value of the hydrogen ion concentration of the solution under investigation. Its influence is most marked from $p_H = 5$ to $p_H = 1·5$, *i.e.* at the range where pure gelatin gels least readily. It is interesting that this range is immediately on the acid side of the iso-electric point. The relationship between sodium chloride content and gelling power is obviously not a simple one and requires further study. One clear point however emerges from the study of Fig. 1, *i.e.* the greater the concentration of neutral salt, the less the influence of the hydrogen ion.

If gelation is regarded as a function of viscosity, the influence of sodium chloride as found by the experiments described here, does not agree with previous work by Mörner [1889] and Freundlich [1909].

The inhibition of setting in strongly acid solutions both in presence and absence of salt can be shown to be due to the hydrogen ion since on neutralisation with sodium hydroxide the gelling power is restored. Neutralisation does not restore gelling power to the strongly alkaline sols. The gelling power in this case is permanently destroyed.

III. *The influence of hydrochloric acid, sodium hydroxide and sodium chloride on the turbidity of sols and gels.*

The observations on turbidity were made in all cases on solutions of gelatin of 1 % or weaker, and refer only to their condition at 15°. 1 % gels of gelatin in water are turbid under these conditions; in 0·1 *N* sodium chloride they are less turbid, in 0·4 *N* sodium chloride they are again slightly less turbid than in 0·1 *N* salt and in normal sodium chloride they are only faintly opalescent. In acid solutions the influence of sodium chloride is reversed. In the absence of sodium chloride the 1 % gelatin solutions in hydrochloric acid are generally clear. A slight turbidity develops on standing if the acid concentration does not exceed 0·005 *N* [Jordan Lloyd, 1920]. The addition of sodium chloride in concentrations of 0·1 *N*, 0·4 *N* and 1·0 *N* causes the clear gels to become turbid. In the last case the gels containing 0·01–0·008 *N* HCl are actually cloudier than those with salt alone. In dilute alkaline solutions (0·002 *N* or less) the addition of sodium chloride causes the clear gels to become turbid. In stronger alkaline solutions (0·05 *N* or more) the addition is without effect, but evidence from other sources shows that the gelatin is actually destroyed at this reaction.

The influence of acid, alkali and salt is summarised in Tables II, III, IV and V. Miss Laing has very kindly examined the turbid water gels for me by means of the ultra-microscope and finds that they contain long fibres similar to the fibres in soap curds.

The relations of the turbidity or "degree of dispersion" of the gelatin to acid, alkali and salt form a close parallel to those for globulin described by Hardy [1905].

Table II.

48 hours at 15°. Salt concentration = 0·00.

Reaction		Concentration of gelatin				
		1·0 %	0·9 %	0·8 %	0·7 %	0·6 %
·25 *N* HCl	$p_H = 0·7$	Clear fluid	—	—	—	—
·20 *N*	0·8	„ soft gel	Clear gelatinous fluid	—	—	—
·15 *N*	0·9	„ „	„ „	—	—	—
·10 *N*	1·1	„ gel	„ „	—	—	—
·05 *N*	1·5	„ „	„ „	—	—	—
·04 *N*	1·6	„ soft gel	„ „	—	—	—
·03 *N*	1·7	„ „	„ „	—	—	—
·02 *N*	2·0	„ fluid	Clear fluid	—	—	—
·01 *N*	2·5	„ „	„ „	—	—	—
·005 *N*	3·8	„ soft gel	„ „	—	—	—
·0025 *N*	4·4	„ „	Clear gelatinous fluid	—	—	—
Water	5·0	Firm opaque gel	Turbid soft gel	Turbid soft gel	Turbid gelatinous fluid	Turbid fluid
·005 *N* NaOH	10·4	Clear gel	Clear gel	Clear gelatinous fluid	Clear fluid	—
·01 *N*	11·7	„ „	„ „	„ „	„ „	—
·02 *N*	12·1	Clear very soft gel	„ fluid	„ —	„ —	—
·03 *N*	12·4	Clear fluid	—	—	—	—
·04 *N*	12·5					
·05 *N*	12·6	„ „	—	—	—	—
·10 *N*	12·9					

Table III.

48 hours at 15°. Salt concentration = 0·10 *N*.

Reaction	Concentration of gelatin 1·0 %	0·9 %	0·8 %	0·7 %	0·6 %	0·5 %
Hydrochloric acid						
p_H = 0·8	Faintly turbid gelatinous fluid	—	—	—	—	—
p_H = 1·1	Faintly turbid gel	Faintly turbid soft gel	Clear fluid	—	—	—
p_H = 1·5	,, ,,	,, ,,	,, ,,	—	—	—
p_H = 2·7	,, ,,	Faintly turbid gel	Faintly turbid soft gel	Clear gel	Clear gelatinous fluid	Clear fluid
p_H = 3·8	,, ,,	,, ,,	Faintly turbid gel	,, ,,	Clear soft gel	,, ,,
Water						
p_H = 5·1	Turbid gel	Turbid gel	,, ,,	Faintly turbid gel	Faintly turbid soft gel	Faintly turbid fluid
Sodium hydroxide						
p_H = 10·4	Faintly turbid gel	,, ,,	,, ,,	Faintly turbid soft gel	Faintly turbid fluid	—
p_H = 11·8	,, ,,	,, ,,	,, ,,	Faintly turbid gel	,, ,,	—
p_H = 12·6	Clear fluid	—	—	—	—	—
p_H = 13·0	,, ,,	—	—	—	—	—

Table IV.

48 hours at 15°. Salt concentration = 0·40 *N*.

Reaction	Concentration of gelatin 1·0 %	0·9 %	0·8 %	0·7 %	0·6 %	0·5 %
Hydrochloric acid						
p_H = 0·8	Faintly turbid fluid	—	—	—	—	—
p_H = 1·1	Turbid gel	Turbid soft gel	Faintly turbid soft gel	Clear fluid	—	—
p_H = 1·5	,, ,,	,, ,,	,, ,,	Faintly turbid gelatinous fluid	Faintly turbid fluid	—
p_H = 2·7	,, ,,	Turbid gel	Faintly turbid gel	,, ,,	Faintly turbid gelatinous fluid	Clear fluid
p_H = 3·8	,, ,,	,, ,,	,, ,,	,, ,,	,, ,,	,, ,,
Water						
p_H = 5·1	,, ,,	,, ,,	,, ,,	Faintly turbid gel	,,	Faintly turbid fluid
Sodium hydroxide						
p_H = 10·4	Turbid gel	,, ,,	,, ,,	Clear soft gel	,, ,,	Clear fluid
p_H = 11·8	,, ,,	,, ,,	Faintly turbid soft gel	Clear gelatinous fluid	Clear fluid	—
p_H = 12·6	Clear fluid	—	—	—	—	—
p_H = 13·0	,, ,,	—	—	—	—	—

Table V.

48 hours at 15°. Salt concentration = 1·0 *N*.

Reaction	Concentration of gelatin 1·0 %	0·9 %	0·8 %
Hydrochloric acid			
p_H = 0·8	Faintly turbid fluid	—	—
p_H = 0·9	Faintly turbid gelatinous fluid	Faintly turbid fluid	—
p_H = 1·1	Faintly turbid soft gel	„ „	—
p_H = 1·5	Faintly turbid gel	Faintly turbid gelatinous fluid	Faintly turbid fluid
p_H = 2·5	Turbid gel	Faintly turbid gel	Faintly turbid gelatinous fluid
p_H = 3·8	„ „	„ „	„ „
Water p_H = 5·1	Faintly turbid gel	„ „	„ „
Sodium hydroxide			
p_H = 10·4	Clear gel	Clear gel	„ „
p_H = 11·7	„ „	„ fluid	—
p_H = 12·1	„ fluid	—	—
p_H = 12·5	„ „	—	—

IV. *Optical rotation.*

The rotatory power of a solution of gelatin is not a constant one but varies with temperature. This property has been examined by Trunkel [1910] and Smith [1919].

Smith considers that above 35° gelatin is converted to a form which he calls gelatin *A* which has not the property of forming gels. Below 15° Smith considers gelatin exists in a form which he calls gelatin *B*, which is the substance capable of gel formation. Between these two temperatures, a solution of gelatin is considered to be a mixture of the two isomers. The conversion of *A* to *B* is reversible. Smith, using the sodium line, gives the following values:

$$[\alpha]_D \text{ at } 35° \text{ and above} = -141°,$$

$$[\alpha]_D \text{ at } 15° \text{ and below} = -313°.$$

The rotatory power of the purified gelatin used throughout these experiments was examined in solutions of distilled water, dilute acid, or dilute alkali. The reaction of the solution in distilled water is approximately $p_H = 5$. It was found that all three solutions showed mutarotation, and that the rotatory power differed in acid, alkaline and distilled water solution. The mercury green line was used as the source of light in all experiments. $[\alpha]_{\text{Hg green}}$ is the rotation caused by a column of fluid 10 cm. in length when each cc. of solution contained 1 g. of pure gelatin. Constant values were obtained for the higher temperature by keeping the tubes for three hours at 37°: at the lower temperature by keeping the tubes for 48 hours at 10° before taking a reading. Intermediate values were not taken. All three solutions set to gels at the lower temperature, the acid and alkaline gels are clear, the water gel cloudy.

For this reason a short tube had to be used in the last case. The following experimental figures were obtained:

Solvent	0·017 *N* HCl	Water $p_H = 5$	0·017 *N* NaOH
Concentration of gelatin	1·67 %	2·00 %	1·67 %
Length of polarimeter tube	18·9 cm.	9·9 cm.	20 cm.
Observed rotation at 37°	−5·03°	−2·81°	−4·97°
Observed rotation at 10°	−9·55°	−6·08°	−9·34°
$[\alpha]_{Hg}$ at 37°	−160°	−142°	−150°
$[\alpha]_{Hg}$ at 10°	−302°	−307°	−280°

These values for $[\alpha]_{Hg}$ at 10° and 37° are reversible. After several heatings and coolings, the value of the alkaline, and to a less extent of the acid tube, tends to sink. The difference in the values of the acid, alkaline and neutral solutions at 37° is a considerable one. A further investigation of the influence of temperature and reaction on the value of $[\alpha]_{Hg\ green}$ is being carried out.

V. *Note on the combination of gelatin with hydrochloric acid and sodium hydroxide and on molecular structure in sols and gels.*

If the gelling power of a gelatin solution be plotted against the reaction, in a system free from inorganic salts, as in Fig. 1, it can be seen that the former is reduced over a range of reaction immediately on the acid side of the iso-electric point, Michaelis and Grineff's [1912] figure being taken for the last point. Over this same range, the gelatin is combining rapidly with hydrochloric acid to form soluble hydrochlorides [Procter, 1914; Loeb, 1919; Lloyd and Mayes, 1922]. On the theory that gels are formed of a framework of uncombined gelatin, penetrated with a solution of soluble gelatin salts, it is possible to suppose that the loss of gelling power at these reactions is due to the reduction of the uncombined gelatin to below the minimum necessary for the formation of a rigid framework. The temporary reappearance of the gel state at higher reactions is probably due to the reduction of the active mass of the water owing to the hydration of the protein ions. At higher p_H values the gel state is again inhibited. Immediately on the alkaline side of the iso-electric point, the presence of free hydroxyl ions has very little effect. Yet it is known that the combination of gelatin with sodium hydroxide begins immediately on the alkaline side of $p_H = 4{\cdot}6$, though the rate of combination is slower by about a third for bases up to a concentration of 0·01 *N* of free hydrogen or hydroxyl ions. At this point on the curve of "base fixed" the curve inflects and this point corresponds to a p_H value of 12 beyond which gelation cannot exist [see Lloyd and Mayes, 1922, Fig. 5]. Smith [1921] finds that the rise of osmotic pressure to a maximum is more rapid in alkaline solutions than in acid. Evidence has been given elsewhere [Lloyd and Mayes, 1922] that acids and bases combine with gelatin at different positions in the molecule and this is supported by the observations given above on the gelling power. It even seems possible that the sodium salts of gelatin may be able to build themselves into the jelly framework in the undissociated state. The loss of gelling power at $p_H = 12$ is to be attributed to actual destruction of

the gelatin molecule in the presence of the strong alkali. Patten and Johnson [1919] also found that liquefaction accompanied high alkalinity ($p_H = 8$, 9 or 10 in their experiments) but as their observations were all made in the presence of buffer salts they are not comparable with those given in this paper. The effect of sodium chloride in diminishing the minimum concentration of gelatin required to form a gel is obscure, but a possibility suggests itself that it may be due to withdrawal of water from the system by the hydration of its ions. This hypothesis is consistent with the fact that sodium chloride increases the turbidity of gelatin in acid or alkaline solution but is difficult to harmonise with the observation that sodium chloride decreases the turbidity of gels near the iso-electric point, and when in sufficient concentration also decreases the setting power. (It may be remarked in parenthesis that Dr Holker has examined the influence of sodium chloride on some of the gelatin used in this work, at a temperature of 40°. At this temperature, according to Smith, the gelatin would be entirely transformed into the non-gelling form. Dr Holker finds that at this temperature the curve of turbidity against sodium chloride content shows the characteristic sine form [Holker, 1921]. This fine variation of turbidity at 40° is however different from the coarser variations described in this paper which are more of the nature of an actual separating out of gelatin from its solutions.)

In a recent paper [Jordan Lloyd, 1920] the theory was put forward that gelatin gels consist structurally of a framework of solid iso-electric or neutral gelatin together with water, through the open meshes of which lies an aqueous solution of ionised gelatin salts, either acid or basic. The solid phase was assumed to be the seat of the elastic forces of the gels, and the liquid phase of the osmotic forces. The necessity for gelatin in both the uncombined, uncharged, and the ionised, charged condition for the formation of a stable gel, was postulated. This theory has been criticised adversely by both Procter [1920] and Laing and McBain [1920] on the grounds that the structure postulated was supposed to be of microscopic dimensions. This, however, is a misinterpretation of the position taken up by the writer. There is no need to assume that the framework, even if solid, is necessarily of an order of magnitude that would be visible under a microscope, and the existence of glass-like gels of gelatin, showing that they are free from particles of an order of magnitude greater than 10^{-6} cm. in diameter, is against this. Adam [1921] has shown that films of palmitic acid on water only one molecule in thickness may either be in the solid or the liquid state. This proves that the solid state is not incompatible with structure of molecular dimensions. The writer does not consider that there is any fundamental difference between the "molecular network" of Procter, the "exceedingly fine filamentous structure" of Laing and McBain, or the "solid framework" postulated by herself, though originally suggested by Hardy. (It is noteworthy that though Hardy made his gelatin frameworks of microscopic dimensions by the arbitrary use of alcohol, he explicitly states in his original paper [1900] that the droplets may be any size

from macroscopic to sub-microscopic according to the conditions under which they separate.) The existence of structure in both gels and sols would seem to have been proved by work on the viscosity of melted gels [see P. van Schroeder, 1903; Garrett, 1903; Levites, 1908; Gokum, 1908; Schorr, 1911; and more recently Hess, 1922], and further the anisotropic properties demonstrated by Hatschek [1921] are evidence that a part at any rate of a gel exhibits the characteristics of a solid. Bearing in mind the work of Adam already referred to, it does not seem impossible that the liquefaction and solidification of gels may be associated with a liquefaction and solidification of the framework similar to the liquefication and solidification of a film of palmitic acid one molecule in thickness.

Procter considers gelatin gels to be of the nature of solid solutions, presumably similar to glass. Laing and McBain state that "a gel is identical with a sol except for its mechanical properties." It is perhaps helpful to consider the matter in the light of the kinetic theory. According to this theory it is possible to visualise the solid state as one in which the molecules composing it are able to vibrate only round a mean position which is fixed, and the fluid state as one in which the molecules (or ions) composing it are free to migrate in any direction. If the molecules of solid are displaced from their mean position by the application of an external force, they will return towards it on the removal of the force, *i.e.* provided that the body has not been strained beyond its elastic limit. Molecules in a liquid displaced by an external force will not return to their previous position on the removal of the force. Gelatin gels are elastic solids with a modulus that has been determined [Leick, 1904], but the classical researches of Graham [1850, 1851] and Dumanski [1907] show that ions can migrate through them as rapidly as through water provided the gel be not too concentrated; *i.e.* experiments on diffusion show that part of the gel at least is fluid and that in gels not too concentrated the fluid phase is continuous. Hardy's observations on the reversal of continuity of phases in the alcohol-water-gelatin system, with increasing concentration of gelatin, have a bearing on this point. Laing and McBain have shown the similarity of gels and sols for other physical properties. The simplest theory harmonising all these facts does seem to be that in sols there is a meshwork of protein in the fluid state through which is an aqueous solution non-miscible with it; in gels the framework is not fluid but solid. The justification for this is to be found in the work of Adam on palmitic acid films. The extension of the theory to suppose that the framework was iso-electric gelatin, and the solution gelatin salts, led to the deduction that perfectly pure gelatin would be unable to form stable gels. This supposition has since been confirmed by the observations of the writer [Jordan Lloyd, 1920]; Field [1921] and Smith [1921].

SUMMARY.

1. Gelatin purified by dialysis and precipitation in alcohol is only soluble with difficulty in boiling distilled water. On cooling, if the concentration be 0·8 % or above, the solution sets to an opaque white gel; if the concentration be 0·7 % or less, the solutions on cooling become turbid fluids. The gels are not stable but show syneresis. The addition of sodium chloride in small quantities (0·4 *N*) *decreases* turbidity and *increases* gelling power, 0·6 % gelatin setting to a soft almost clear gel at 15° in 48 hours in the presence of 0·1 *N* sodium chloride: at 1·0 *N* concentration sodium chloride *decreases* turbidity and *decreases* gelling power.

2. In the presence of hydrochloric acid, gelatin becomes much more readily soluble. Sols and gels are transparent to the naked eye. Hydrochloric acid diminishes the gelling power, the degree of diminution passing through a maximum between the reactions $p_H = 2$ and $p_H = 3$, and again beyond $p_H = 0·7$ at 15°. The addition of sodium chloride in small quantities (0·1 to 1·0 *N*) *increases* turbidity and *increases* gelling power up to a p_H of 0·9. In stronger acid solutions the effect of salt is to decrease gelling power. The presence of the sodium chloride does not appreciably affect the reaction of the system.

3. In the presence of caustic soda, gelatin is more soluble than in water. Sols and gels are transparent to the naked eye. Caustic soda slightly decreases the gelling power between $p_H = 10$ and $p_H = 12$ and completely prevents gelation at concentrations above $p_H = 12$. At concentrations $p_H = 12$ and above slow hydrolysis occurs. The addition of sodium chloride in small quantities (0·4 *N*) *increases* turbidity and *increases* gelling power. In greater concentrations (1·0 *N*) gelling power is decreased. The presence of the sodium chloride is without appreciable effect on the reaction of the system.

4. Gelatin therefore can exist in the forms clear sol, clear gel, turbid sol, turbid gel, at the same temperature and the same concentration.

5. Aqueous solutions of pure gelatin are not precipitated by the salts of the heavy metals.

6. The rotatory power of gelatin is not a constant but varies (*a*) with temperature, (*b*) in acid, neutral or alkaline solutions.

7. The bearing of the above properties on the theory of gelation is discussed.

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